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RESPIRATORY ENZYMES

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Preface

The First Edition of "Respiratory Enzymes" was a collection of seminar reports in a field which was comparatively new to biochemists in this country. Much of the material has long since become out of date in the light of newer knowledge which has been rapidly accumulated in this field. Yet the demand for a text covering this special aspect of enzymology seemed to justify a revision. In the present edition the authors have been assigned subjects with which they are familiar by virtue of research experience. An attempt has been made to avoid duplication of material in the various chapters but beyond this the content, emphasis and style are those of the individual authors.

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Chapter I

HISTORICAL INTRODUCTION

by

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INTRODUCTION

The subject of respiratory enzymes naturally deals with those enzymes catalyzing the chemical reactions which take place in respiration. It is well to deal first with respiratory enzymes rather than cellular respiration because less is known of the differences in respiration between various types of cells or different parts of the same cell. A general knowledge of the respiratory enzymes is needed before the respiration processes in the various tissues can be studied successfully.

We are all familiar with enzymes and the general properties of enzymes. One fact which should be kept in mind is that an enzyme cannot alter the equilibrium of a chemical reaction; it can only increase the rate at which the equilibrium is reached. Thus we must recognize the potential difference as a driving force in the reaction as well as activation of the reactants by the enzyme.

In order to limit our discussion, it is necessary to specify what is meant by respiration. The broadest conception of respiration involves all chemical reactions in living cells which release energy. This would include fermentation and would carry a discussion of respiratory enzymes back to the time of Berzelius 1837, who first included fermentation under catalytic processes. However, it is better to limit our discussion to aerobic respiration and to leave anaerobic metabolism or fermentation to a separate chapter. In other words, the more generally accepted definition may be used: Respiration is the aggregate of those processes by which oxygen is introduced into the system and carbon dioxide is removed.

We should also specify that respiratory enzymes deal only with the reactions within the living cell. This eliminates the voluminous literature concerned with the interchange of respiratory gases. However, it is now known that an enzyme, carbonic anhydrase, is concerned in gaseous exchange. We may use the term "desmolyzing enzymes" for those enzymes concerned in fermentation and respiratory enzymes for those involved in biological oxidation or in respiration.

True respiration, therefore, is concerned with oxidation of metabolites and the release of energy. The release of energy is of value in maintaining the temperature of the cell, but maintenance of body temperature is not important in all aerobic organisms. The energy used for other purposes may be the more important, and we should keep in mind that the complexity of the enzyme systems is probably very closely associated with the energy requirements for different purposes. Living tissues are not dependent so much upon mere release of energy as on the controlled release of energy.

HISTORICAL

The necessity of continuous oxygen supply for living matter was first described by Lavoisier in 1770. Of course the relation of the atmosphere to the life of an animal was recognized before the work of Lavoisier. Many of the investigators held that the vital factor in the relationship was the mechanical movement of the lungs. In 1667 Hook demonstrated that a dog could be kept alive without movement of this organ if air were blown into the lungs. Mayow in 1674 suggested that respiration and combustion were similar because both processes removed the same thing from the air, but his work was lost sight of. When Lavoisier demonstrated the importance of oxygen, he believed that the oxidation took place in the lungs. It was as late as 1880 before it was universally accepted that organic molecules are oxidized in the tissues. Today we know that the tissues are the seat of all respiration and that when the organisms are too large for their cells to obtain oxygen by simple diffusion from the air, mechanisms for supplying oxygen have been evolved. In the insects, for example, we have the tracheal system and in higher animals the lungs or gills in conjunction with the circulatory system. We also recognize that the most important function of hemoglobin is that of carrying oxygen from the lungs to the tissues where the oxidation takes place. It is important to emphasize that the oxygen liberated from hemoglobin at the tissues is not in an activated form but is merely atmospheric oxygen.

RESPIRATORY ENZYMES

Since most metabolites can be left in contact with the air for indefinite periods without undergoing any appreciable change, while in the tissues they are readily oxidized, we must assume that the tissues contain a number of enzymes which are together responsible for tissue respiration. These enzymes have been known by the general name of oxidases. The term "oxydase" was first used by Bertrand (1896). A number of respiratory enzymes have been extracted from tissues and studied separately. The efficiency of some of these is truly remarkable. For example, succinic acid, which will withstand boiling nitric acid, is rapidly oxidized by atmospheric oxygen in the presence of an aqueous suspension of liver.

Before discussing the known individual respiratory enzymes let us first consider the early investigations along this line. As early as 1860 workers suggested that certain tissues contained catalysts of oxidation but it was not until 1900 that these were thought of as enzymes.

$$\text{Reducer} \xleftarrow{\text{Catalyst}} \text{Oxidizer}$$

The catalyst may activate either the reducer or the oxidizer. The early question was, is protoplasm a strong reducing system or an oxidizing system? Today we know that both mechanisms are present; when one molecule is reduced another is oxidized.

The early workers were agreed that O_2 was activated in all the reactions. They all used the peroxidase reagents for the detection of active oxygen because colored products were formed. No color was obtained with oxygen but in the presence of activated oxygen the guaiacum darkened immediately. Most of the tests were made on plant materials rather than on animal tissues.

The above work stimulated interest in substances such as phosphorus, benzaldehyde, essential oils, etc., which undergo spontaneous oxidation at low temperatures. This was called autoxidation and was soon associated with coupled oxidation, i.e. oxidation of a second substance in the presence of the first. For example, turpentine undergoes autoxidation, methylene blue is rather stable, but if the two are put together both compounds are oxidized.

The real study of biological oxidations started with Schonbein who worked between 1850 and 1865. His work, as well as the results of subsequent investigation is carefully reviewed by Kastle (1910) in "The oxidases and other oxygen-catalysts concerned in biological oxidations." Schönbein discovered ozone in 1840 and found that ozone was produced during the autoxidation of phosphorus. He suggested three allotropic forms of oxygen:

Neutral O Ozone ⊖ Antozone ⊕

In autoxidation the oxygen is split into: ⊖ which oxidizes the metabolite, ⊕ which forms H_2O_2 from H_2O. He thought of ozone as anything which would oxidize guaiacum. He found that H_2O_2 would not oxidize guaiacum but that plants and animals contained something which would bring about this oxidation. He said that the tissues changed the antozone to ozone ⊖ which was active. Today we know of the substance which promotes oxidation in the presence of hydrogen peroxide as a peroxidase. We know that tissues also contain catalase which decomposes hydrogen peroxide into water and oxygen. Schönbein, therefore, demonstrated the existence of a catalyst and suggested peroxide formation although he was not aware of peroxidase and catalase as separate enzymes. He looked for the catalyst in blood because he thought the oxidation took place there.

Hoppe-Seyler in 1878 suggested the following system for the rupture of the oxygen molecule:

$$AH_2 + O\text{-}O \longrightarrow A + H_2O + \text{active O used for oxidizing other material.}$$

The following reaction was used as an example:

$$PdH_2 + O_2 + \text{indigo} \longrightarrow Pd + H_2O + \text{oxidized indigo.}$$

Bach showed that this reaction could be resolved into two stages:

$PdH_2 + O_2 \longrightarrow Pd + H_2O_2$ Pd removed, then Indigo + $H_2O_2 \longrightarrow$ oxidized indigo + H_2O.

Ehrlich in 1885 thought there was a giant living molecule with side chains which stored oxygen as peroxides. During oxidation this molecule was disrupted by certain stimuli. It is now well known that cells cannot store extra oxygen.

Traube in 1885 studied the autoxidation of zinc.

$$Zn + \begin{matrix} OH\text{-}H \\ OH\text{-}H \end{matrix} + \begin{matrix} O \\ O \end{matrix} \longrightarrow Zn(OH)_2 + H_2O_2$$

Two new points were established as a result of this work:

1. The importance of water in oxidation and reduction.
2. The direct reduction of oxygen to hydrogen peroxide.

Bach and Engler independently in 1897 suggested the formation of organic peroxides which were able to oxidize other compounds.

$$A + O \longrightarrow A\begin{matrix} O \\ | \\ O \end{matrix} \qquad A\begin{matrix} O \\ | \\ O \end{matrix} + A' \longrightarrow AO + A'O$$

This idea dominated the field for 20 years despite the fact that these workers had very little evidence for the formation of organic peroxides. The only peroxide isolated was that of benzaldehyde and this may have been formed from H_2O_2 and benzaldehyde.

Bach and Chodat in 1903 extended this theory. They worked mostly on vegetable material and their theory was quite generally adopted. They resolved the positive guaiacum test given by plants into two parts.

$$A + O_2 \xrightarrow{\text{Oxygenase}} A\begin{matrix} O \\ | \\ O \end{matrix} \qquad A\begin{matrix} O \\ | \\ O \end{matrix} + A' \xrightarrow{\text{Peroxidase}} A'O + AO$$

The peroxide then oxidized other compounds. Their conception of the mechanism of the oxidase action was as follows: The oxygenase is capable of forming an organic peroxide in air. A peroxidase will then catalyze the decomposition of the peroxide formed by the oxygenase and the active oxygen is accepted by guaiacum.

Wheldale-Onslow (1911) pointed out that plants which show the direct oxidase test, that is, which blue guaiacum without H_2O_2, contain a substance giving the catechol reaction while no such substance was found in those plants giving the peroxidase test. She showed that a catechol solution itself autoxidizes in air with the production of peroxide. Later she showed that only compounds with the orthodihydroxy grouping autoxidize and that such compounds are present in oxidase plants and are accompanied by oxygenase. This system was found to be absent from peroxidase plants. The enzyme was separated from the substrate with alcohol and was active when recombined with catechol.

Onslow and Robinson (1925) showed that a preparation of oxygenase oxidized compounds with a monophenolic grouping, such as cresol and hydroxy benzoic acids, to dihydroxy compounds. At the same time Szent-Györgyi (1925) suggested that orthobenzoquinone was the product of oxidation of catechol by oxygenase and was solely responsible for the blueing of guaiacum. He allowed the oxygenase to work on catechol, then removed the enzyme with alcohol and found the orthoquinone blued guaiacum apart from any enzyme action. In 1926 Onslow and Robinson confirmed this work but found that the enzyme could not be removed completely. They also demonstrated the presence of H_2O_2 in autoxidized solutions of catechol as well as in solutions of catechol oxidized with oxygenase.

RESPIRATORY ENZYMES

In 1927 Pugh and Raper through the isolation of crystalline aniline derivatives confirmed the formation of orthoquinone from phenol, p-cresol and catechol by the action of an oxidizing enzyme isolated from mealworm, and termed by them, tyrosinase; also the formation of orthoquinone from catechol by action of peroxidase and H_2O_2. In 1928 Onslow and Robinson suggested that the secondary oxidation of monophenol is due to the presence of orthoquinone.

The presence of H_2O_2 in addition to orthoquinone greatly complicates the picture. Raper (1928) has pointed out that the direct guaiacum reaction cannot be used as an indication of one particular enzyme. All the following oxidation systems gave this reaction, and the components may and do occur together in many plant tissues.

1. Peroxidase with an autoxidizable substance which produces peroxide in air.
2. Peroxidase with an aerobic dehydrogenase such as xanthine oxidase which produces H_2O_2.
3. Peroxidase with polyphenol oxidase in the presence of a polyphenol.
4. Polyphenol oxidase with catechol or derivatives. This gives rise to an orthoquinone which oxidizes guaiacum directly.
5. Tyrosinase with tyrosine. Orthoquinone is produced.

It is clear that a positive oxidase reaction with guaiacum does not show the presence of any particular oxidizing enzyme or system although it does indicate that peroxidase, tyrpsinase, or a polyphenolase may be present. This reaction will not distinguish between them, nor does it necessarily indicate the presence of a dual oxygenase-peroxidase system of the Bach-Chodat type.

Work on the following subjects has given us much of the early information about respiratory enzymes: Dehydrogenases, Molecular Oxygen as a Hydrogen Acceptor, Peroxidase and Catalase, Warburg's 'Respiratory Enzymes', Glutathione, Cytochrome, Indophenol Oxidase. Much of the material included in the following discussion was taken from a review by Dixon (1929) and the papers on cytochrome by Keilin (1925-30).

DEHYDROGENASES

One of the most important subjects of investigation in the field of respiratory enzymes has been the study of the dehydrogenases. Dehydrogenases are enzymes which activate the hydrogen of the metabolites. The reducing power of tissues was first noticed by histologists. Ehrlich in 1885 injected methylene blue into tissues of animals and found some tissues reduced methylene blue while others did not. Of course this result is dependent upon the balance between the oxidation and the reduction taking place in the tissue at any moment.

The real significance of reducing systems was first realized in 1914. Before this Schardinger (1902) had found an enzyme in milk which would reduce methylene blue in the presence of an aldehyde. Bach tried to explain the action of this enzyme by his peroxide theory but was unsuccessful. The next important step was the work of Wieland (1922). He believed that oxidation could go on in the absence of oxygen and that the great majority of oxidations were dehydrogenations. In those cases where there was a gain in oxygen, Wieland assumed a preliminary hydrate formation. Thus in the case of aldehydes the following reactions occurred:.

$$\text{-}\overset{\text{H}}{\underset{|}{\text{C}}}\text{=O} + H_2O \longrightarrow \overset{\text{H}}{\underset{|}{\text{C}}}\text{-OH} \qquad\qquad \overset{\text{H}}{\underset{|}{\text{C}}}\text{-OH} + B \longrightarrow \text{-C=O} + BH_2$$
$$\text{OH} \qquad\qquad\qquad\qquad \text{OH}$$

In proof of this reaction Wieland quoted the fact that chloral dissolved in benzene cannot reduce silver oxide, whereas chloral hydrate in benzene was able to do so.

By the use of palladium black Wieland was able to resolve the oxidation-reduction reaction into two successive stages.

$$AH_2 + Pd \longrightarrow A + PdH_2$$

$$PdH_2 + B \longrightarrow Pd + BH_2$$

On adding palladium black to an aldehyde solution the aldehyde became oxidized to the acid, while the palladium became charged with hydrogen. The palladium was oxidized with atmospheric oxygen or methylene blue.

The application of this view to oxidations in animal tissues was greatly advanced by the work of Thunberg. He demonstrated, by means of the methylene blue technique, that animal tissues contain respiratory enzymes capable of activating a number of organic substances. Methylene blue is an active reducible dye which can be used conveniently as the oxidizer in biological systems. The rate at which the blue color disappears is an indication of the rate at which the oxidation takes place. Since the colorless MbH_2 is readily reoxidized to methylene blue by atmospheric oxygen the reactions must be studied in special vessels which can be evacuated (Thunberg tubes).

Thunberg added methylene blue to suspensions of minced animal tissues in neutral buffer solutions and determined the effect of adding various organic substances on the reduction time. Fresh animal tissues rapidly reduced methylene blue without the addition of any oxidizable substance, since they contained many substances activated by the dehydrogenases. Thunberg found, however, that by a preliminary extraction of the tissue with water, these substances could be removed; the tissue then lost the power of reducing the dye. Most of the dehydrogenases were left intact in the tissue and the reducing power could be restored by adding substances capable of being activated by them.

By such means Thunberg demonstrated the presence in frog muscle of dehydrogenases capable of bringing about the oxidation of the following:

Lactic acid - $CH_3CHOHCOOH$

Oxybutyric acids - α - and β -$CH_3CH_2CHOHCOOH$, $CH_3CHOHCH_2COOH$

Succinic acid - $COOH(CH_2)_2COOH$, but not glutaric acid - $COOH(CH_2)_3COOH$

Malic acid - $COOH\ CH_2CHOH\ COOH$

Citric acid - $COOH\ CH_2.COHCOOH.CH_2\ COOH$

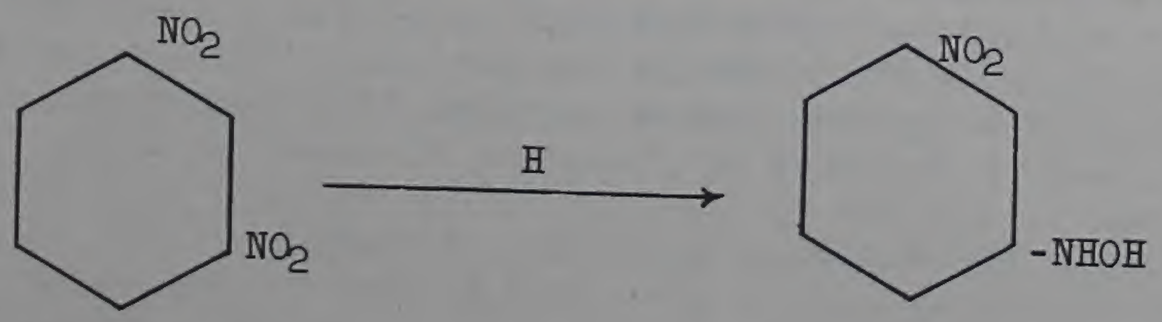
Tartaric acid - $COOH\ CHOH\ CHOH\ COOH$

Glutamic acid - $COOH\ CH_2.CH_2.CH\ NH_2.COOH$; not other amino acids

Glycerophosphoric acid but not glucose

Dehydrogenases have since been found to be very widely distributed, not only in all animal tissues, but also in plant tissues, yeast cells, bacteria, etc.

Thunberg's work on frog muscle was repeated by Lipschitz in 1921 using m-dinitrobenzene as an oxidizer instead of methylene blue. He obtained exactly the same result.



m-dinitrobenzene

m-nitrophenyl hydroxylamine

Four of the dehydrogenases received attention in the early studies; namely, those responsible for the oxidation of succinic acid, purine bases and aldehydes, lactic acid, and citric acid.

MOLECULAR OXYGEN AS A HYDROGEN ACCEPTOR

We now come to the important question of whether activation of the substance to be oxidized is alone sufficient to secure its oxidation by molecular oxygen, or whether an activation of the oxygen is also necessary. The evidence suggests that oxygen can act without being activated in some of the systems but in others it cannot. Of course, in the presence of methylene blue, the active hydrogen can be taken up directly by molecular oxygen. For instance, the reduced form of methylene blue is directly oxidized by molecular oxygen even in the presence of cyanide which is known to inhibit oxygen-activating systems.

When we turn to the dehydrogenase systems we find that in some cases the same condition applies. In the oxidation of xanthines and of aldehydes activation by the dehydrogenase is sufficient to render them readily oxidizable by molecular oxygen, and there is much evidence to show that no special oxygen-activating catalyst or carrier is concerned in this system. The addition of cyanide has no effect and the oxygen appears to be equivalent to the methylene blue as a hydrogen acceptor.

In other cases this does not apply, e.g., the lactic and citric dehydrogenases. Here the activated molecules, which are freely oxidized by methylene blue, are quite unable to react with molecular oxygen. The reason for this important difference was unknown for some time.

The case of the succinic dehydrogenase is particularly interesting. As mentioned before, a suspension of succinic dehydrogenase plus succinate rapidly absorbs oxygen and reduces methylene blue. The addition of a very small amount of cyanide, however, completely abolishes the oxygen uptake while it leaves the reduction of methylene blue unaffected. The cyanide clearly cannot be preventing the activation of the succinate for then it would affect the methylene blue reduction. Therefore, oxygen must be unable to react with activated succinate molecules and the cyanide acts by inactivating either some carrier or catalyst particularly concerned with oxygen in the solution. The nature of this other system has been made clear by Keilin and we will discuss his work in detail later. Dixon and Thurlow found that the oxidation of hypoxanthine in liver was completely unaffected by the addition of cyanide which, in the same sample of tissue, completely prevented the oxygen uptake due to succinate.

On the basis of the failure of the lactic dehydrogenase system, and similar systems to react with oxygen, Szent-Györgyi proposed the theory that active hydrogen reacts only with active oxygen. This view is not wholly accepted because it ignores those systems which do not require active oxygen.

The dehydrogenases may be divided into two classes. Aerobic dehydrogenases, such as xanthine dehydrogenase, which can use molecular oxygen directly and anaerobic dehydrogenases, such as lactic dehydrogenase, which can utilize oxygen only by the cooperation of some other system.

DEHYDROGENASE-PEROXIDASE SYSTEMS

Thurlow (1925) showed the relation between the products formed by dehydrogenases and the peroxidases in the cells. We have already seen that one class of dehydrogenases can utilize oxygen as a hydrogen acceptor and from other facts presented it would be expected that hydrogen peroxide would be formed in the process. Thurlow, working on the xanthine and aldehyde dehydrogenase systems, actually found that considerable quantities of H_2O_2 were produced and could be detected by a variety of chemical tests.

$$(E)\ X.O.H_2 + O_2 = (E) + XO + H_2O_2$$

Thurlow found that when hypoxanthine or xanthine was added to a suspension of xanthine dehydrogenase and shaken with air, peroxide could very soon be detected in the solution and, as the oxidation proceeded, the amount of peroxide present rapidly increased. After some time a maximum was reached but then the amount of H_2O_2 gradually decreased due to its reaction with the xanthine.

Now according to Bach's definition of an "oxygenase" as a system capable of converting oxygen into a peroxide, it will be seen that such a dehydrogenase plus its substrate is an oxygenase. Thurlow showed that this was the case. On adding guaiacum to an aerated solution containing xanthine dehydrogenase plus hypoxanthine plus (animal or vegetable) peroxidase a deep blue color was at once produced.

Thurlow found that for studying the system it was preferable to use nitrite in the place of guaiacum. In the presence of animal peroxidases hydrogen peroxide readily oxidized nitrite to nitrate and the amount of nitrite remaining unoxidized could be determined colorimetrically. Thurlow used peroxidases prepared from liver or milk. The following table gives the typical results:

Tube	Contents	Nitrite unoxidized
1	Xanthine dehydrogenase + xanthine	++++
2	Xanthine + peroxidase	++++
3	Xanthine dehydrogenase + peroxidase	++++
4	Xanthine dehydrogenase + xanthine + peroxidase	-

Thus no oxidation took place except in Tube 4 where all the constituents were present and here the nitrite was rapidly and completely oxidized.

Szent-Györgyi has confirmed these results using adrenaline, which became oxidized to a colored product, instead of nitrite. Harrison and Thurlow showed that the complete system also brought about the oxidation of some of the ether soluble substance in milk. These reactions are true coupled systems because only one molecule of nitrite can be oxidized for each molecule of xanthine oxidized.

PEROXIDASE AND CATALASE

Peroxidase was first recognized as a specific enzyme by Linossier in 1898. Much of the early work was done on the vegetable peroxidases. The work of Willstätter on the purification of vegetable peroxidases is classical.

There has been much controversy over the relation of iron to peroxidase. The results obtained by Willstätter with horse-radish peroxidase suggested that it was not an iron compound. However, the action of peroxidase closely resembles that of organic iron compounds related to hematin. The relation of free iron is also interesting. Fenton in 1894 first showed the activating action of ferrous iron on H_2O_2. Dakin in 1922 showed that many of the oxidations known to occur during metabolism can be brought about by H_2O_2 plus a trace of ferrous salt.

Harrison and Thurlow showed that ferrous iron, hematin, etc. were capable of acting as coupled systems with xanthine oxidase; they found indeed that with iron, instead of the peroxidase which they prepared from milk, the system was able to oxidize lactic acid and β-hydroxybutyric acid which it was unable to do if the milk peroxidase were used. On the other hand there were indications that the true peroxidase may act on substances which were not acted upon by hematin or iron. It was also interesting that with the iron the lactic acid was not oxidized to pyruvic acid but to acetaldehyde.

The peroxidases have been studied almost entirely by the use of peroxidase reagents. This is regrettable since the peroxidase reagents are merely substitutes for the substances normally oxidized and the result is that we have very little knowledge as to the substances actually oxidized by them in vivo.

Catalase was discovered by Loew in 1901. Hydrogen peroxide is extremely toxic to most of the respiratory systems and it is the function of catalase to keep the concentration of H_2O_2 down by decomposing it to water and oxygen. The recent work on catalase will be discussed later.

WARBURG'S THEORY AND THE "RESPIRATORY ENZYME"

It is now time to consider a theory of cell respiration which has assumed considerable prominence in the history of respiratory enzymes. Warburg's theory is, in brief, that respiration or the utilization of molecular oxygen by the living cell is a process which is due to catalysis by iron. He has summarized the essential point of the theory in the following words: "According to the theory the catalyst of respiration is iron.....The primary reaction of respiration is the reaction between molecular oxygen and iron, and molecular oxygen can only react in the cell by this reaction, and not with the organic molecules." See Warburg (1928) Dixon (1928), and Anson and Mirsky (1930).

This would definitely exclude the possibility of molecular oxygen acting as a direct acceptor for the hydrogen in accordance with the Wieland view. The two theories are definitely contradictory, as Warburg pointed out, in respect to the manner in which molecular oxygen acts. Wieland regarded the activation of the hydrogen as the important event, while Warburg considered the activation of the oxygen by the iron as the essential process.

According to the Warburg view, the uptake of oxygen by the cell is to be regarded not as the sum total of the separate uptakes of a number of distinct oxidase systems, but as a process due to the action of one catalytic mechanism. He says unity in the cells and multiplicity in the extracts are not contradictory, because the various oxidases which have been isolated are merely odd bits derived from the main enzyme.

With regard to the nature of the catalytic iron, Warburg stated that the free ions are inactive and that the active iron is present in the form of a definite complex compound. This catalytic compound, whose action is responsible for respiration as a whole, Warburg calls "the respiratory enzyme" (das Atmungsferment); and as a result of later work he believes it to be a compound related to the hematin series of pigments.

According to Warburg's first theory, organic molecules reduced the oxidized form of iron which could in turn be oxidized by atmospheric oxygen. The iron acted merely as an intermediate carrier of oxygen. This process is often referred to as oxygen activation but it can be nothing but iron catalysis.

The more recent theory of Warburg assumes that the respiratory enzyme combines with oxygen as follows:

$$XFe + O_2 = XFeO_2$$

The oxidation of the organic molecules then follows thus:

$$XFeO_2 + 2A = XFe + 2AO$$

This view which involves an actual oxygen activation resembles the theory of Bach in many ways.

A brief summary will be given of the evidence upon which Warburg's theory is based. In 1921 he studied the inhibitory effect of a large number of narcotic substances on the velocity of cell respiration. Among other substances alcohols, ketones, urethanes, amides, substituted ureas, etc. were used. As the result of a large number of measurements he came to the conclusion that the inhibitory power of these substances was due to their adsorption on the active surfaces where respiration takes place. One or two substances, however, exerted inhibitory powers out of all proportion to their adsorption. HCN and H_2S were such substances. HCN in a concentration of M/10,000 has definite inhibitory effects on most cells and this effect is about 10,000 times what would be predicted from its adsorption. The substances which acted thus were known to inhibit most reactions catalyzed by iron.

Next Warburg studied the behavior of a "cell model". He found that a suspension of blood charcoal (charcoal made by heating blood) in water was capable of oxidizing, by means of molecular oxygen, many substances especially oxalic acid and certain amino acids. The action of narcotics and cyanide was found to be closely similar to their action on the cell.

Blood charcoal naturally contained a certain amount of iron and Warburg believed that the catalytic power of the charcoal was due to the presence of iron. He confirmed this view by preparing iron-free charcoal by heating a mixture of pure cane sugar and silicate. This proved to be quite inactive. If iron salts were added to the mixture before heating the charcoal was still inactive, but if iron and a nitrogen compound were added the resulting charcoal was very active. Warburg concluded that the activity was not due to the charcoal surfaces, but to the iron-nitrogen-carbon complexes in that surface and that HCN acted by uniting with the iron and thus destroying the active centers. He believed that the same condition was present in cells because he found that a small amount of cyanide completely abolished the respiration of sea urchin eggs, yeast cells and bird's red blood corpuscles.

Warburg found that carbon monoxide also inhibited cell respiration and his evidence for the relationship of the "respiratory enzyme" with hematin arises chiefly from work on this effect. It was well known that carbon monoxide formed addition compounds with many iron compounds related to hematin and that these compounds were dissociated by light. Warburg found that the marked inhibition of the respiration of yeast produced by CO was almost entirely abolished by light. He made use of the effect of light to determine the spectrum of the CO compound of the "respiratory enzyme". By determining the effectiveness of light of different wave lengths in restoring the respiration inhibited by CO, he was able to obtain a curve showing how the effectiveness varied with the wave length. The natural assumption was that light must have been absorbed by the CO-respiratory enzyme if it were to be effective in dissociating it, and in that case the curve must represent the actual absorption spectrum of the compound. The curve obtained closely resembled the spectrum of CO-hemochromogen. This was the main reason for believing that the respiratory enzyme was a derivative of hematin.

Thus Warburg believed that all oxidations are catalyzed by this iron compound. He admitted that such oxidations as that of reduced methylene blue are true autoxidations but denied that natural substances are autoxidizable. In support of this denial he cited two cases of apparent autoxidations which he had shown to be due to iron catalysis. One of these was the oxidation of fructose in phosphate solutions which was shown by Meyerhof and Matsuoka to be dependent upon traces of iron in the system. The reaction was greatly accelerated by the addition of traces of iron or copper and strongly inhibited by cyanide. The other was the case of the oxidation of substances which contained the sulphydryl group, such as cysteine and glutathione. The oxidation of these compounds had been thought of as autoxidation and considerable importance had been ascribed to it in connection with biological oxidations but it was shown by Warburg and Sakuma that this was also iron catalysis. They found that pure preparations of cysteine were not autoxidizable but became so upon the addition of traces of metals. These experiments were repeated on glutathione by Harrison who obtained similar results.

At this point let us consider some of the main criticisms which have been leveled against Warburg's theory. Warburg's claim that his enzyme accounted for the whole respiration of animal tissues cannot be allowed because Dixon and Elliott have shown that most tissues are inhibited only to an extent of 40 to 60 per cent by HCN. It is, therefore, clear that this enzyme can only account for 60 per cent of the respiration at the most. Since xanthine oxidase is an aerobic dehydrogenase and is not inhibited by cyanide, the oxidation brought about in the presence of this enzyme cannot be due to an iron catalysis.

With regard to the spectrum of the CO compound of the "respiratory enzyme", it seems doubtful whether Warburg's interpretation can be accepted. Keilin pointed out that in photo-chemical reactions the presence of a colored substance, the so-called sensitizer, was frequently necessary. The function of this substance was simply to absorb the light and make the energy available for the reaction. Now in such systems it was found that the effective light corresponded simply with the absorption spectrum of the sensitizer. It was possible that in the dissociation of the CO compound of the "respiratory enzyme" the ordinary hematin compounds of the cell act as sensitizers and the curve observed is merely

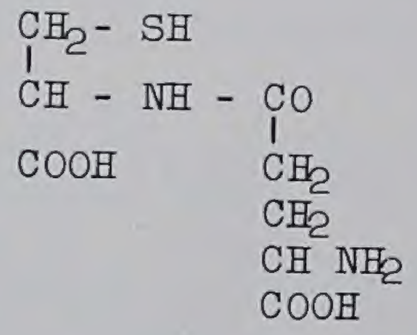
their spectrum and not that of the enzyme. A number of reasons for believing that the enzyme was not a hematin compound will be given later.

Finally it must not be forgotten that cyanide poisons many systems besides the "respiratory enzyme", for instance peroxidases, polyphenol oxidase and related oxidases, catalase, the glutathione system, etc. It does not follow that the sole effect of the cyanide inhibition is due to its action on Warburg's enzyme.

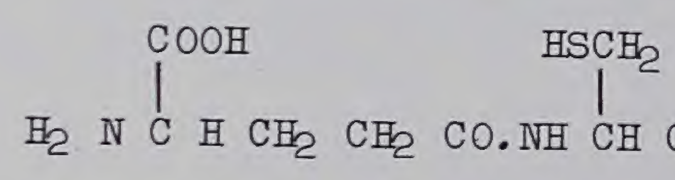
These points of criticism leave the main point, the existence and importance of the "respiratory enzyme", unaffected. It is clear that a large part of the respiration is due to a catalyst which behaves like an iron compound and is poisoned by HCN, CO, H_2S, etc. It is also clear that other types of systems may contribute a by no means negligible part of the respiration.

GLUTATHIONE

Before continuing the discussion on the relation of oxidases to Warburg's theory let us consider the relation of glutathione to the mechanism of respiration. De Ray Pailhade in 1885 noted that there was some material in cells which reduced sulphur. He called it philothione. Heffter in 1908 found that tissues gave the nitroprusside test. Arnold found that practically all tissues gave this test. Heffter said that the reducing power of tissues was due to the SH groups, but today we know that the reducing power is due to the dehydrogenases. Meyerhof in 1917 claimed that the SH compounds were connected with tissue respiration. Glutathione was isolated by Hopkins (1921) who showed that it was very widely distributed in living cells. He then believed it to be a dipeptide containing cysteine and glutamic acid. The following formula for glutathione was worked out by Quastel, Stewart and Tunnicliffe (1923).



Tunnicliffe and Stewart (1925) attempted to prove this formula through synthesis but more recent work demonstrated glutathione to be a tripeptide with the following formula.



H_2 N C H CH_2 CH_2 CO.NH CH CO.NH CH_2 COOH; glutamyl cysteinyl glycine.

As it is the sulphur group alone which is of importance in connection with tissue oxidations, its formula may be abbreviated to GSH.

It readily undergoes oxidation to a disulphide form thus:

$$2GSH + B = G.S.S.G. + BH_2$$

Interest in this material arose mainly from the fact that cells contain systems both which vigorously reduce oxidized glutathione and which rapidly oxidize reduced glutathione by means of molecular oxygen. For some time glutathione was the only autoxidizable material which had been extracted from animal tissues.

Although reduced glutathione is rapidly oxidized in the tissues, glutathione exists in the reduced form. This is because GSSG is continually being reduced; if GSSG is added to tissues it is reduced. One naturally thinks of the dehydrogenase systems as responsible for this reduction. This, however, is incorrect since none of the known dehydrogenase systems is capable of reducing glutathione in the slightest degree (Hopkins and Dixon, 1922) (glucose dehydrogenase is an exception). It seems that enzyme systems are not involved in

the reduction for the reaction is unaffected by heating the tissue to the boiling point. Washed muscle heated to the boiling point and extracted with alcohol was still able to reduce oxidized glutathione vigorously and was called a "thermostable preparation". An aqueous suspension of such a preparation was unable to absorb oxygen but on the addition of a little disulphide glutathione an oxygen uptake was established at once. It was shown that the disulphide form was reduced by the "fixed SH groups" apparently forming part of the protein structure, which were not soluble in water and which were unable to react directly with oxygen or methylene blue. These groups became oxidized by reducing the added disulphide and the reduced glutathione formed then reacted with the oxygen.

We say that glutathione undergoes autoxidation but this is not strictly true because it is similar to cysteine. Mathews and Walker studied the autoxidation of cysteine. In 1923 Warburg and Sakuma showed that the apparent autoxidation was in fact due to small traces of catalytic metals (iron and copper) present as impurities. They showed that absolutely metal-free cysteine solutions absorbed oxygen only at a negligible rate, that the addition of iron or copper enormously accelerated its oxidation and that its oxygen uptake was abolished by adding a little KCN which inactivated the metals. These observations were confirmed and extended to glutathione by Harrison (1924). The iron probably unites with cysteine and forms a compound which is alternately oxidized and reduced. Cremer has shown that CO will unite with the iron compound of cysteine. These facts, of course, do not affect the position of glutathione as far as its biological action is concerned, as in any case the tissues contain the iron necessary to make the glutathione autoxidizable.

Upon studying the "thermostable system" further it was found that the amount of the disulphide form reduced was equivalent to the fixed SH groups present, but the amount of oxygen taken up by the system was much larger and amounted to several times the equivalent of the SH groups originally present. The extra oxygen thus taken up was in fact utilized by the system for oxidation of the proteins present. The mechanism of this protein oxidation is not yet clear, but it appears to be linked in some way with the oxidation of the sulphydryl group of glutathione, and is thus dependent ultimately upon the fixed SH groups which maintain the glutathione in the reduced form. Instead of using the tissue preparation other proteins containing SH groups may be used; denatured egg albumin which contains SH groups may be oxidized with glutathione but natural egg albumin is not.

Hopkins has also shown that the reduced form of glutathione will catalyze the autoxidation of unsaturated fats and fatty acids, particularly in acid solution. The mechanism of this catalysis is still unknown.

All of this work was done when glutathione was considered to be a dipeptide of cysteine and glutamic acid. Hunter and Eagles (1927) threw some doubt on this structure and suggested that serine might also be a constituent of the molecule. Johnson and Voegtlin (1927) published an apparent confirmation of the original view of Hopkins. This led Hopkins (1929) to reinvestigate his early work and he succeeded in finding a method for the ready isolation of glutathione in the pure crystalline state. He then found that the substance was a tripeptide containing cysteine, glycine and glutamic acid. Kendall, McKenzie and Mason (1929) have confirmed this work. The old preparation was undoubtedly impure and contained a large amount of the tripeptide but probably contained free cysteine in addition. Meldrum and Dixon have repeated much of the early work using the new crystalline glutathione instead of the old impure material. Some of their results are very interesting.

Solutions of the crystalline material absorb oxygen at a slow rate. This rate, unlike that of cysteine or the impure material, cannot be accelerated by the addition of iron.

They suggest that the autoxidation of glutathione depended upon the cooperation of two factors, namely, iron or copper and some substance able to form catalytically active complexes with the metals. With a crystalline glutathione the rate of oxidation was limited by the amount of the second factor present and not by the iron.

The crystalline material, unlike the old preparations, produced no oxygen uptake when added to thermostable tissue preparations. When the disulphide form was added it became reduced by the tissue but no oxygen uptake resulted. When the sulphydryl form was added not only did it fail to oxidize the protein but its own autoxidation was completely inhibited.

The solutions were reactivated by the addition of a small amount of the old material. It was probable that the tissue inactivated the glutathione by removing the impurity which formed active complexes with metals. Free iron and copper were inactive in the oxidation of glutathione and the glutathione was unable to form active complexes with them. It followed that pure reduced glutathione was not autoxidizable even in the presence of iron or copper.

A mixture of a crystalline glutathione and free cysteine behaved in the same way as the earlier glutathione preparations. Thioglycollic acid may also bring about the oxidation. However, quantitative experiments did not support the view that the material in the impure glutathione was only one of these two compounds.

The almost universal distribution of glutathione shows that it must be of some vital importance to the cell. Yet the glutathione content of any one tissue has not so far been correlated with the predominance or comparative absence of any one type of metabolism. The respiration of yeast may be reduced by 60% to 90% by narcotics, which inhibit the reducing systems, and by cyanide, which prevents the activity of the indophenol oxidase, without the amount of reduced glutathione being affected. Yeast may be starved until its rate of respiration falls to about 5% of that in glucose solution without affecting the thiol content. The part played by glutathione in the life of the cell is still obscure. It is possible that it is connected with protein metabolism as has been suggested by Waldschmidt-Leitz.

CYTOCHROME

We now come to one of the most fascinating stories in the history of biochemistry - the discovery of cytochrome by Keilin. Perhaps we should say the interpretation of cytochrome rather than discovery, because MacMunn in 1886 had demonstrated that a pigment with four spectroscopic bands, which he called myohematin, was present in certain cells especially those with a high rate of metabolism. He realized that this pigment was related to hemochromogen, but was unable to explain the exact relation or to account for the fact that this compound had four bands while hemochromogen had only two.

Our knowledge of this pigment was not expanded until Keilin started his painstaking work in 1925. He introduced the term cytochrome and demonstrated by means of spectroscopic observations that it existed in most living cells. The spectrum of cytochrome is, for instance, very intense and easy to observe in the extremely active wings of bees which can contract several hundred times a second. It is also easily detected in striated muscles of mammals and birds and in baker's yeast. Keilin has demonstrated its presence in almost 100 different materials including many of the lower animals such as the Molluscs and Crustacea, in 40 species of insects, in various tissues of higher animals, in bacteria as well as yeast, and in plants.

Cytochrome can be oxidized and reduced under suitable conditions. In the reduced condition it shows a characteristic absorption spectrum with four bands, the positions of which in all organisms are approximately the same: 6019, 5665, 5502, and 5210 A. In the oxidized form there are no clear absorption bands but only faint shading at 520-540 and at 550-570. Cytochrome is not a single four banded pigment but a mixture of three different hemochromogens. Three of the bands are the α bands of the three hemochromogens, the fourth band is the result of the fusion of the β bands of the three hemochromogens. Keilin's reasons for believing that it is not a single substance are these: The relative intensities of the bands vary from the cytochrome of one tissue to that of another. When it is oxidized and reduced, the four bands do not disappear and reappear simultaneously. Extraction will remove one of the components before the others.

The precise nature of the iron-porphyrin nuclei of the three components of cytochrome has not been definitely decided upon, but some evidence has been obtained which suggests that there are two different hematins in cytochrome, one similar to that present in hemoglobin and the other similar to the hematin of chlorocruorin. This result was obtained by extracting yeast with pyridine to remove the unknown nitrogenous substances. Two different pyridine hemochromogens were thus obtained, one like that derived from hemoglobin and the other similar to that derived from chlorocruorin. Fischer has obtained coproporphyrin and

Kammerer's porphyrin from yeast. These two distinct porphyrins are probably derived from the two hemochromogens that make up the cytochrome of yeast. Keilin has succeeded in producing from ordinary pyridine hemochromogen a pigment or a mixture of pigments with a spectrum similar to that of cytochrome. He believed his procedure to consist partly in the modification of the porphyrin by oxidation and reduction, and partly in the precipitation of the pigment.

Before considering the function of cytochrome, the universal distribution of this pigment should be emphasized. For example, when Keilin took a section 3-6 mm. thick from various bulbs, potatoes, grains of wheat, barley, oats, or stamens of young flowers, treated them with a reducer such as sodium hydrosulphite and examined them under a microspectroscope the spectrum of cytochrome could be observed. In other words, hematin compounds were found in plants as well as in animals. It was formerly believed that animals built their hematin compounds from the chlorophyl which they obtained from plants. But it must now be accepted that the hematin compounds are more universal than either hemoglobin or chlorophyl. The rapidity with which growing yeast can manufacture cytochrome is truly remarkable. Hemoglobin is merely a specialized derivative of hematin, but it is an exceptional derivative because it is the only hematin compound which has the property of forming a loose combination with oxygen.

It has been mentioned that cytochrome can be oxidized and reduced under suitable conditions. Under natural conditions in the living cells cytochrome is in the oxidized or in only partially reduced form. The condition of cytochrome as seen spectroscopically in the living organism denotes only the state of equilibrium between the rate of its oxidation and reduction at that particular time. If yeast is well aerated, its cytochrome is oxidized. If yeast is exposed to an atmosphere of nitrogen, its cytochrome is reduced. In the case of insects, when the animal is active, the cytochrome is partially oxidized; when the insect is quiescent, the cytochrome is again reduced.

When a drop of a weak solution of KCN is added to a suspension of yeast, the cytochrome remains completely reduced no matter how actively this suspension is shaken with oxygen and continues to show the characteristic bands of reduced cytochrome. A concentration of N/10,000 KCN will stop the oxidation of cytochrome. Furthermore, if a drop of KCN is added to a suspension of yeast, kept at a low temperature, and previously oxidized by a current of air, the cytochrome becomes immediately reduced, just as if the cyanide were acting as a powerful reducing agent. Actually, KCN does not act as a reducer but inhibits the oxidation of the cytochrome without affecting the reactions causing the reduction of the pigment.

Other substances such as formaldehyde, ethyl alcohol, acetone, and urethane act in a very different way. All these substances, even in concentrations which kill the yeast cells, do not inhibit the oxidation of cytochrome. On the contrary, they completely stop the reduction of cytochrome and it remains oxidized indefinitely. If a suspension of yeast is treated with urethane and shaken with air until all the cytochrome is oxidized and then treated with KCN, the cytochrome does not become reduced. It remains oxidized because the reducing action of the cells is inhibited. These facts clearly indicate that there are two mechanisms in the cells where cytochrome functions in their metabolism, one which has to do with the oxidation of cytochrome, and a second which controls the reduction of this pigment. Cytochrome must, therefore, act as an intermediate carrier between the catalytic systems which reduce it and those which oxidize it, and its degree of oxidation at any instant will depend simply on the relative velocities with which the two reactions take place. Any factor which diminishes the activity of the reducing systems will send the cytochrome over to the oxidized state, while factors which inhibit the oxidizing systems will cause it to become more reduced.

Keilin's later work (1929) was concerned with the properties and identification of these two systems and incidentally with the correlation of the systems we have discussed previously. He found that the systems responsible for the reduction of cytochrome in the tissues to be the dehydrogenases as we would expect from what has already been said. On the other hand, the catalyst responsible for the oxidation by atmospheric oxygen proved to be identical with the indophenol oxidase which is so widely distributed in animal tissues and yeast. In general, then, all factors which inhibit the indophenol oxidase inhibit the oxidation of cytochrome.

Dehydrogenases are inhibited by narcotics but not by cyanide and these facts fit into the reactions already observed. Further, by washing the tissues with water, the dehydrogenases become inoperative because their substrates are removed and washing prevents the reduction of cytochrome. The activity of the dehydrogenases may be restored by adding various hydrogen donators and these also restore to washed tissue their power to reduce cytochrome. This may be easily demonstrated by using a sample of heart muscle which has been minced and washed; there is no tendency for the cytochrome in this tissue to become reduced. However, if a small amount of succinate is added the cytochrome becomes reduced in about 15 seconds due to the activity of the succinic acid dehydrogenase. This reduction is in opposition to the oxidizing action of the indophenol oxidase, and if this is abolished by the addition of a little cyanide before the succinate is added, the reduction of the cytochrome is instantaneous. It is clear then that in the absence of cyanide a continuous reduction of the cytochrome by the dehydrogenase and oxidation by the oxidase must be going on resulting in a continuous uptake of oxygen and oxidation of the succinate. Keilin's work, therefore, provides the explanation for the effect of cyanide on the succinic dehydrogenase system which was previously somewhat puzzling. We can conclude then that the succinic dehydrogenase, like the lactic and citric dehydrogenases, is unable to react with molecular oxygen and that the oxygen must come from the cytochrome. The latter is activated by indophenol oxidase and therefore the entire reaction may be inhibited by cyanide.

INDOPHENOL OXIDASE

The work of Keilin (1929) indicates that indophenol oxidase is one of the most important oxidases. It was necessary for him to demonstrate the actual presence of this enzyme in yeast which he had used in most of his studies.

In 1885 Ehrlich had shown that intravenous injection of α-naphthol and paraphenylenediamine resulted in the formation of indophenol. Rohmann and Spitzer (1895) made use of equal amounts of α-naphthol and paraphenylenediamine for studying the oxidizing power of various animal tissues. Today the Nadi reagent which consists of dimethyl-paraphenylenediamine and α-naphthol is more commonly used for the detection of indophenol oxidase.

Schönbein (1863) reported that yeast did not blue guaiacum in the presence of H_2O_2. Harden and Zilva (1914) found peroxidase in washed, dried and acetone yeast. However, they could not detect the oxidase. Bach (1916) confirmed this conclusion. Kastle (1909) suggested that reducing systems in muscle and brain retarded tests for the oxidizing systems. This was incidentally obtained with yeast. Keilin found that when the yeast was heated in a buffer solution at 52° for $1\frac{1}{2}$ hours, the activity of the dehydrogenase systems was decreased and the test for the oxidase could be determined without difficulty. Through the use of this method he was able to demonstrate that yeast contained a powerful indophenol oxidase system which was thermostable at 70° and which was strongly inhibited by KCN and H_2S. Warburg found that CO inhibited respiration. Keilin found that the activity of the oxidase was inhibited by high partial pressures of CO in the dark and that the inactive CO-oxidase compound was dissociated when exposed to light with the liberation of the active oxidase. These results together with other more detailed studies indicate that indophenol oxidase is identical with Warburg's Atmungsferment.

A more complete discussion of the individual respiratory enzymes together with all the recent work will be given in the following chapters. However, before leaving the historical developments, it should be emphasized that most of the early work in the field was extremely accurate and that each phase of the work made a definite contribution to our knowledge. Even two such divergent views as those of Warburg and Wieland can find a place in the complete picture as more knowledge accumulates. This fact should be kept in mind as one studies the recent results, some of which seem to be quite conflicting in nature.

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Chapter II

KINETICS AND MECHANISMS OF ENZYME REACTIONS

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KINETICS AND MECHANISMS OF ENZYME REACTIONS

The necessity of a theoretical foundation for integration of the mass of confusing experimental findings in enzyme chemistry has encouraged efforts to apply physical-chemical theories to such studies. The inadequacies of any theory based on the relatively small amount of exact quantitative data available at present is evident to any who makes a critical examination. Although obviously these attempts are foredoomed to only limited success, the experimentalist genuinely interested in increasing our understanding of enzyme activity will acquaint himself with the major aspects of the work designed to furnish a suitable theory for the practice. He then will be in a better position to aid in their eventual reconstruction.

It is not feasible to attempt a complete survey of the numerous publications available if the result is to be anything more than a mere review. Detailed discussion will be made, therefore, only of material believed to represent concepts that have already demonstrated their usefulness or show promise of doing so. This choice allows adequate development of the fundamental theory as a background for the more extensive treatment represented by the bibliography. The plan of the chapter is to discuss first how changes in the physical-chemical environment affect enzyme reactions and how such effects are related mathematically to the rate of the reaction (kinetics). The next section deals with refinements that have been introduced as more and diverse observations became available. The third part treats of possible mechanisms of enzyme action derived from the kinetic considerations. The final section provides a few examples of how specific reaction rates of enzyme systems have been calculated from the kinetic data.

REACTION VELOCITY AND THE PHYSICAL-CHEMICAL ENVIRONMENT

A fundamental property of enzyme systems is that the rate of the reaction depends on the physical and chemical characteristics of the environment, for example, on the pH, oxidation-reduction potential, temperature, concentration of substrate and presence of inhibitors. Although each experimenter usually determines these, empirically at least, to define the "optimum" conditions for subsequent manipulations, few realize that the measurements may have theoretical implications. In the succeeding sections we shall discuss in particular the implications for the theory of enzyme reaction of the measurements made on the effects of temperature, pressure, substrate and inhibitor. Subsequent chapters consider other influences of the physical-chemical environment.

TEMPERATURE

In 1889 Arrhenius empirically suggested the following relationship between rate of reaction and the temperature at which a chemical reaction occurs:

(1) $\frac{d \ln k}{dT} = \frac{A}{T^2}$, i. e., the rate of change of the natural log of the velocity constant of a chemical reaction is inversely proportional to the square of the absolute temperature. The same relationship (equation 2) has been derived in a more exact form from theoretical considerations based on statistical mechanics. (See "A Symposium on the Kinetics of Reaction," Chemical Reviews 17: 43-136, 1935, for discussion of the physical-chemical considerations treated in this and subsequent sections which involve chemical kinetics.)

(2) $\frac{d \ln k}{dT} = \frac{E}{RT^2}$

k = velocity constant of reaction.
T = absolute temperature.
R = gas constant.
E = energy of activation of molecules, i.e., the kinetic energy which must be possessed by a molecule before the reaction will occur.

Integrating this equation,

$$(3)\ \log k = \frac{-E}{2.303\ R}\ \left(\frac{1}{T} + C\right)$$

Equation 3 states that if log k is plotted against the reciprocal of the absolute temperature, the slope of the resulting "best" straight line (best in the least square sense) is equal to $\frac{-E}{4.606}$ calories. The energy of activation, E, can thus be determined from experimental data.

Arrhenius demonstrated that his empirical relationship also held for certain reactions catalyzed by enzymes, e.g., the hydrolysis of sucrose by saccharase. The form for biological reactions is usually written:

$$(4)\ \log k = \frac{-\mu}{2.303\ R}\ \left(\frac{1}{T} + C\right)$$

μ is variously termed the critical increment of temperature, the temperature characteristic or the temperature velocity constant. It is used instead of E to indicate that there is no necessary implication of a physical meaning involved, e.g., "energy of activation."

Crozier (1924) analyzed the values of μ for numerous diverse biological reactions including the following: locomotion of insects and other lower forms of life, frequency of chirping of crickets and katydids, rate of flashing of fireflies and heart-beat of frogs. Unexpectedly, the values obtained had a tendency to cluster about certain figures, viz., 8,000, 12,000 and 18,000 cal. The constancy of the values suggested that a governing reaction plays a similar role in each of these biological responses, e.g., H_2-transfer to oxygen by the cytochrome system or a common dehydrogenation reaction.

The constancy of the value of μ also suggests the basis for the empirical generalization that all biological reactions have a temperature coefficient, $Q_{10} = 2 - 3$, i.e., within their range of activity, the rate of respiration, of growth, or of any other vital phenomenon doubles (or triples) for each 10° rise in temperature. From equation (4) it follows that,

$$(5)\ \log k_2/k_1 = \frac{\mu}{2.303\ R}\ \frac{T_1 - T_2}{T_1 T_2}$$

in which k_1 and k_2 are velocity constants at the absolute temperatures T_1 and T_2. Now if μ equals ca. 12,000 cal. and T_1 and T_2 are 10° apart in the neighborhood of 300° K., e.g., 295° K and 305° K, then

$$\log k_2/k_1 = \frac{12{,}000}{4.6}\ \times\ \frac{10}{295 \times 305} = 0.29$$

k_2/k_1 equals about 2.0 which means that the rate of reaction has been doubled. Similarly, if μ equals 16,500 cal., $\log k_2/k_1$ equals 0.40, and k_2/k_1 equals 2.5.

It may appear surprising that a relatively small increase in temperature causes such a large increase in the rate of reaction. Calculation of the number of molecules with an energy content in excess of a given quantity (energy of activation) discloses the reason for this somewhat unexpected result. Integration of the Maxwell-Boltzmann's distribution equation (Taylor, 1931) reveals that the fraction of the total molecules of a gas with energy content greater than a given value, E, is given by the relation,

$$(6).\ \frac{n'}{n} = e^{-\frac{E}{RT}}$$

n' = number of molecules with an energy greater than E.
n = total number of molecules present.

If the energy of activation equals 12,000 cal. and T equals 300° K (27° C.),

$$\frac{n'}{n} = e^{-\frac{12,000}{2 \times 300}} = e^{-20} = 10^{-20 \times \log e} = 10^{-8.686} = 2.06 \times 10^{-9}$$

and at 310° K (37° C.) $\frac{n'}{n} = e^{-\frac{12,000}{2 \times 310}} = e^{-19.355} = 10^{-8.4058} = 3.93 \times 10^{-9}$

Thus the average kinetic energy of the molecules has increased only 3 per cent $(\frac{10}{300})$, but the fraction of the molecules with energy greater than 12,000 cal. has practically doubled.

Temperature coefficients find practical use in comparing different preparations of the same enzyme to determine if the source of an enzyme alters its properties; for example, Sizer (1937) investigated the effect of source of invertase on the temperature characteristic of the hydrolysis of sucrose. These coefficients also are used for differentiation between similar enzymes when they are present together, such as different types of dehydrogenases in a suspension of Escherichia coli (Gould and Sizer, 1938).

CONCENTRATION OF SUBSTRATE

The effect of the concentration of substrate on the velocity of an enzyme catalyzed reaction is shown in Figure 1.

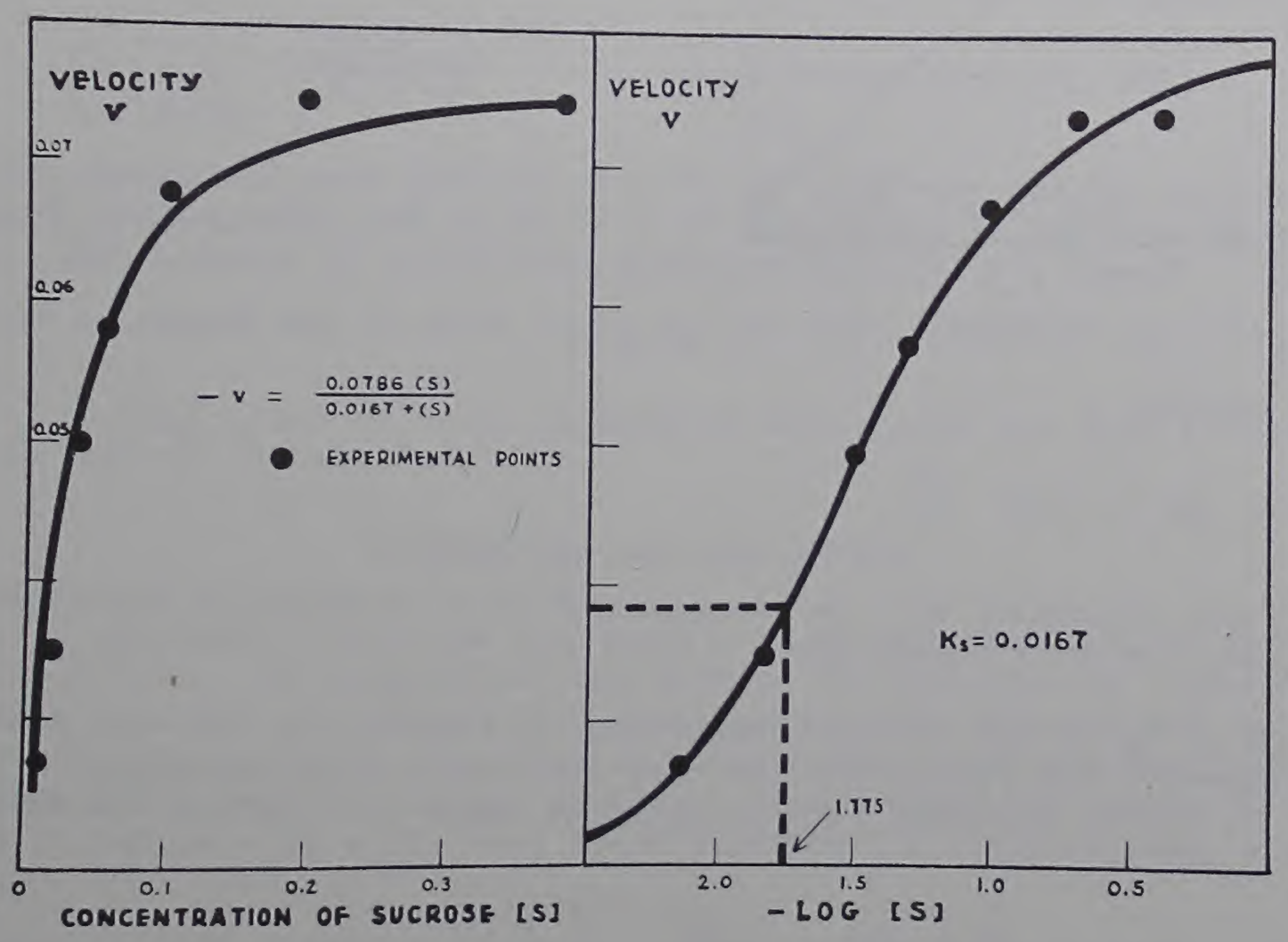


Figure 1

TYPICAL SUBSTRATE CONCENTRATION - VELOCITY CURVE

Data of Michaelis and Menten (1913) on hydrolysis of sucrose by invertase. Right hand portion of graph illustrates method used by Michaelis and Menten to evaluate constants.

A satisfactory mathematical analysis of this type of curve was first made by Michaelis and Menten (1913).[1] The foundation of their treatment is the assumption that the enzyme and substrate form an intermediate which dissociates into free enzyme and products of the reaction. Their derivation follows:

Let the reactions be assumed: Enzyme + Sucrose $\longleftrightarrow$ Enzyme-Substrate Complex $\longrightarrow$ Enzyme + Fructose + Glucose

$E + S \longleftrightarrow ES \xrightarrow{H_2O} E + P$ where P represents products of the reaction.

(E)	concentration of <u>total</u> enzyme.	Then concentration of <u>free</u> enzyme is <u>total</u> less <u>bound</u> or (E) — (ES).
(S)	" " substrate (S)$\rangle\rangle$(E)	
(ES)	" " enzyme-substrate complex	

The dissociation constant of the enzyme-substrate is:

$$K_s = \frac{\text{conc. free enzyme x conc. substrate}}{\text{conc. intermediate}}$$

$$(7)\quad Ks = \frac{[(E) - (ES)]\,(S)}{(ES)} \quad \text{or} \quad (8)\quad (ES)\,Ks + (ES)\,(S) = (E)(S)$$

Solving for (ES)

$$(9)\quad (ES) = \frac{(E)(S)}{Ks + (S)}$$

Now if the <u>velocity constant</u> for the break-up of ES is <u>k</u>, and the measured velocity of the reaction is <u>v</u>, then since the concentration of H_2O is constant, <u>v</u> depends only on the concentration of enzyme-substrate complex or:

$$v = k\,(ES) \text{ and from equation (9) } v = \frac{k\,(E)(S)}{Ks + (S)}$$

The maximum velocity of the reaction, <u>V</u>, will be reached when the concentration of the substrate is so high that all of the enzyme is tied up in the intermediate complex, <u>i.e.</u>, when (E) = (ES). Then V = k (E); substituting this value in equation (9), equation (10), $v = \frac{V\,(S)}{Ks + (S)}$ is obtained. This is the usual form of the Michaelis-Menten equation, but it can be written

$$(10')\quad Ks = (S)\,\frac{V}{v} - 1$$

Since Ks and V are constants this is the equation of a rectangular hyperbola which is the typical form observed (see Figure 1).

Estimation of K_s by the method illustrated in Figure 1 is somewhat cumbersome and inaccurate. Lineweaver and Burk (1934) pointed out that if the reciprocal of both sides of equation (10) is taken, a linear form is obtained which is superior for determination of the values of V and Ks.

$$(11)\quad \frac{1}{v} = \frac{Ks + (S)}{V\,(S)} = \frac{Ks}{V}\left[\frac{1}{(S)}\right] + \frac{1}{V}$$

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[1] Euler has independently derived a similar relationship; the equation is, therefore, often termed the Michaelis-Euler equation. The discussion in this section will deal primarily with the thermodynamic relations of the substrate-enzyme intermediate hypothesis. Interesting suggestions with respect to the actual mechanisms by which the enzyme activates the substrate when combination has occurred have been advanced by Quastel (1926), Wolff (1931), and Taylor (1938).

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(a) $E + S \rightleftharpoons ES \xrightarrow{k} E + P$

(b) $E + I \rightleftharpoons EI$ (inactive)

Concentration of free enzyme is (E) - (ES) - (EI)

(E) - total concentration of enzyme
(S) - concentration of substrate
(I) - concentration of inhibitor
(ES) - concentration of enzyme-substrate complex
(EI) - concentration of enzyme-inhibitor complex
Ks - dissociation constant of ES
Ki - dissociation constant of EI

$$Ks = \frac{[(E) - (ES) - (EI)]\ (S)}{(ES)} \quad \text{and} \quad Ki = \frac{[(E) - (ES) - (EI)]\ (I)}{(EI)}$$

$$(EI) = \frac{(I)\ [(E) - (ES)]}{Ki + (I)} = \frac{(E)\ (I) - (ES)\ (I)}{Ki + (I)}$$

$$Ks\ (ES) = (S)\ \left\{(E) - (ES) - \frac{[(E)(I) - (ES)\ (I)]}{Ki + (I)}\right\}$$

$$= (S)\ \left\{\frac{(E)\ Ki + (E)\ (I) - (ES)\ Ki - (ES)\ (I) - (E)\ (I) + (ES)\ (I)}{Ki + (I)}\right\}$$

$$= (S)\ \left[\frac{(E)\ Ki - (ES)\ Ki}{Ki + (I)}\right]$$

$$(ES)\ [KsKi + Ks(I)] = (E)\ (S)\ Ki - (ES)\ (S)\ Ki$$

$$(ES)\ [KsKi + Ks(I) + (S)\ Ki] = (E)\ (S)\ Ki$$

$$(ES) = \frac{(E)\ (S)\ Ki}{KsKi + Ks(I) + Ki(S)}$$

Now as before $v = k(ES)$ where k is velocity constant of reaction (a)

$$v = \frac{k(E)\ (S)\ Ki}{KsKi + Ks(I) + Ki(S)}$$ and $V = k(E)$ where V is maximum velocity at enzyme saturation.

$$v = \frac{V(S)Ki}{KsKi + Ks(I) + Ki(S)}$$

Taking the reciprocal of both sides -

$$1/v = \frac{KsKi + Ks(I) + Ki(S)}{V(S)Ki} = \frac{KiKs}{V(S)Ki} + \frac{Ks(I)}{V(S)Ki} + \frac{Ki(S)}{V(S)Ki}$$

$$1/v = \frac{Ks}{V(S)} + \frac{Ks(I)}{V(S)Ki} + \frac{1}{V}$$

$$= \frac{1}{V(S)}\ \left[Ks + \frac{Ks(I)}{Ki}\right] + \frac{1}{V} \quad \text{or} \quad (13) \quad 1/v_i = 1/V^* \left[Ks + \frac{Ks(I)}{Ki}\right]\ 1/(S) + 1/V^*$$

in which v_i indicates the velocity and V* the maximum velocity in the presence of inhibitor.

Comparison of equation (13) with equation (11) shows that the effect of a competitive inhibitor is to increase by the quantity $\frac{Ks(I)}{Ki}$ the slope of the line obtained when 1/v is plotted against 1/(S). Hence competitive inhibition is indicated by an increase in the slope of the 1/v : 1/(S) plot accompanied by no significant change in intercept (see Figure 3B). In this figure the "best" straight line for the points corresponding to a given value of the pH_2 was determined by the method of least squares. It was demonstrated that the slopes of the lines differed significantly, but the intercepts did not, two neces-

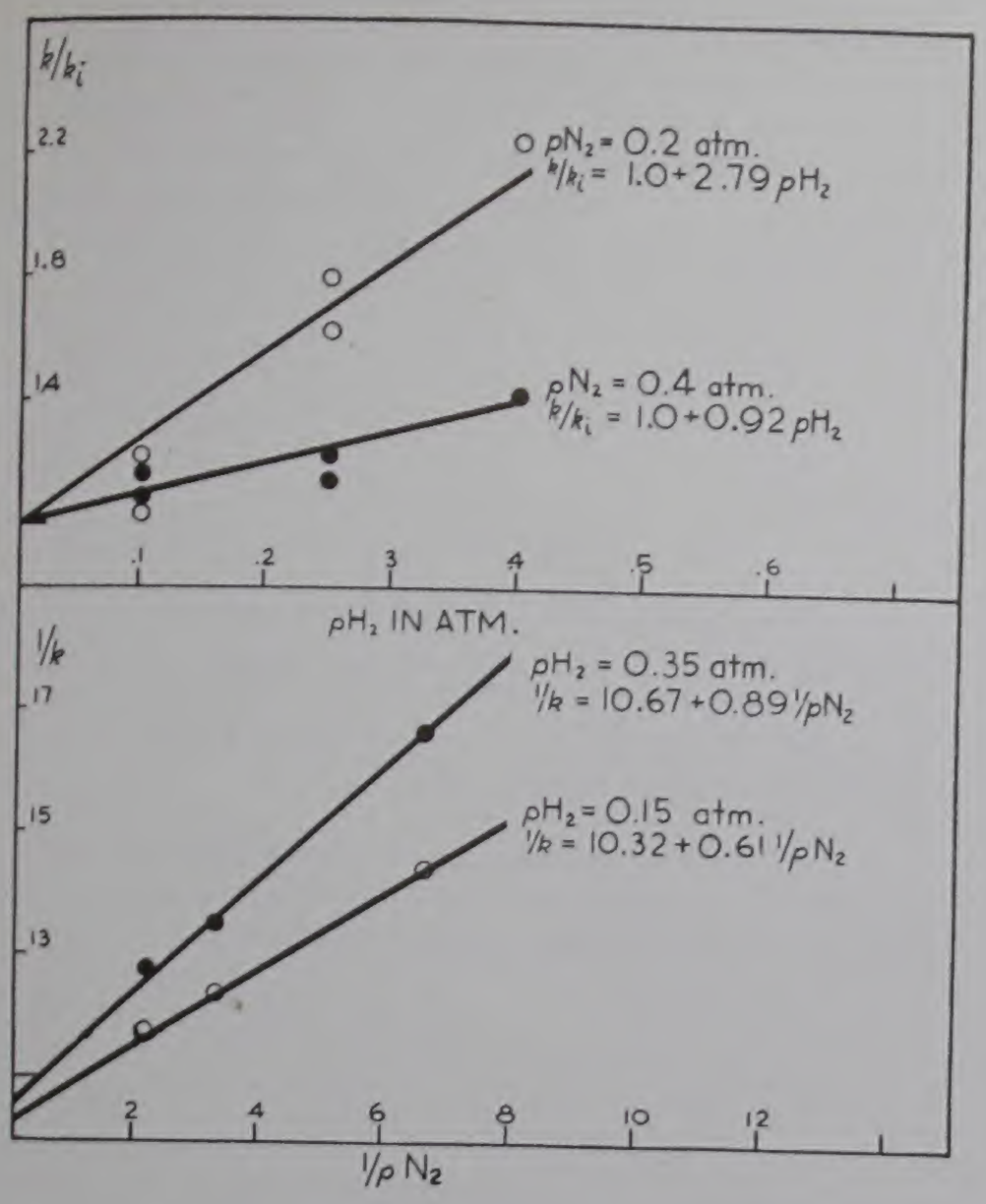


Figure 3

Competitive inhibition of nitrogen fixation in red clover by hydrogen (Wilson, 1940).

A. v/v_i: (I) plot (Top)

B. $1/v$: (S) plot (Bottom)

The significantly different slopes of the lines indicate that the inhibition is competitive.

sary conditions for competitive inhibition. Values of Ki (the dissociation constant of the enzyme-inhibitor complex) were calculated from the relationship, $Ks + \frac{Ks\ (I)}{Ki} = V^*$ times slope of line. Wilson (1940) has estimated the Ks for this system as 0.05 atm. Examination of the expression for determination of Ki reveals that, unless the slope, the intercept, Ks and (I) are known with a rather high degree of accuracy, relatively large errors may enter into the estimate of Ki.

From equations (11) and (13) the alternative form

$$(14)\quad v/v_i = 1 + \frac{Ks}{Ki}\left[\frac{(I)}{Ks + (S)}\right]$$

can be derived. Accordingly, if v/v_i is plotted against different values of inhibitor concentration, (I), straight lines result with unit intercept and a slope dependent on (S) as is illustrated in Figure 3A.

When the inhibition is non-competitive, the reactions may be written:

$E + S \longleftrightarrow ES \longrightarrow E + P$

$E + I \longleftrightarrow EI$ (inactive with dissociation constant, K_i)

$ES + I \longleftrightarrow ESI$ (inactive with dissociation constant, $K_{esi} = K_i$)

By the same type of reasoning and algebraic manipulations already illustrated, the final velocity equations are obtained:

$$(15)\quad 1/v_i = \left[1 + \frac{(I)}{K_i}\right]\left[\frac{1}{V^*} + \left(\frac{K_s}{V^*}\right)\frac{1}{(S)}\right]$$

$$(16)\quad v/v_i = 1 + (I)/K_i$$

Comparison of equation (15) with (11) makes it evident that the effect of a non-competitive inhibitor is to increase both slope and intercept by the factor $[1 + (I)/Ki]$. Competitive inhibition is thus readily differentiated from non-competitive since in the former the intercept does not increase (compare Figures 3A and 4). Equation (16) states that plotting v/v_i against (I) results in a straight line with unit intercept and a slope of $1/K_i$ independent of (S). This difference contrasts with the behavior of a competitive inhibitor in which the slope varies with (S) and thus provides a second means for distinguishing between the two types.

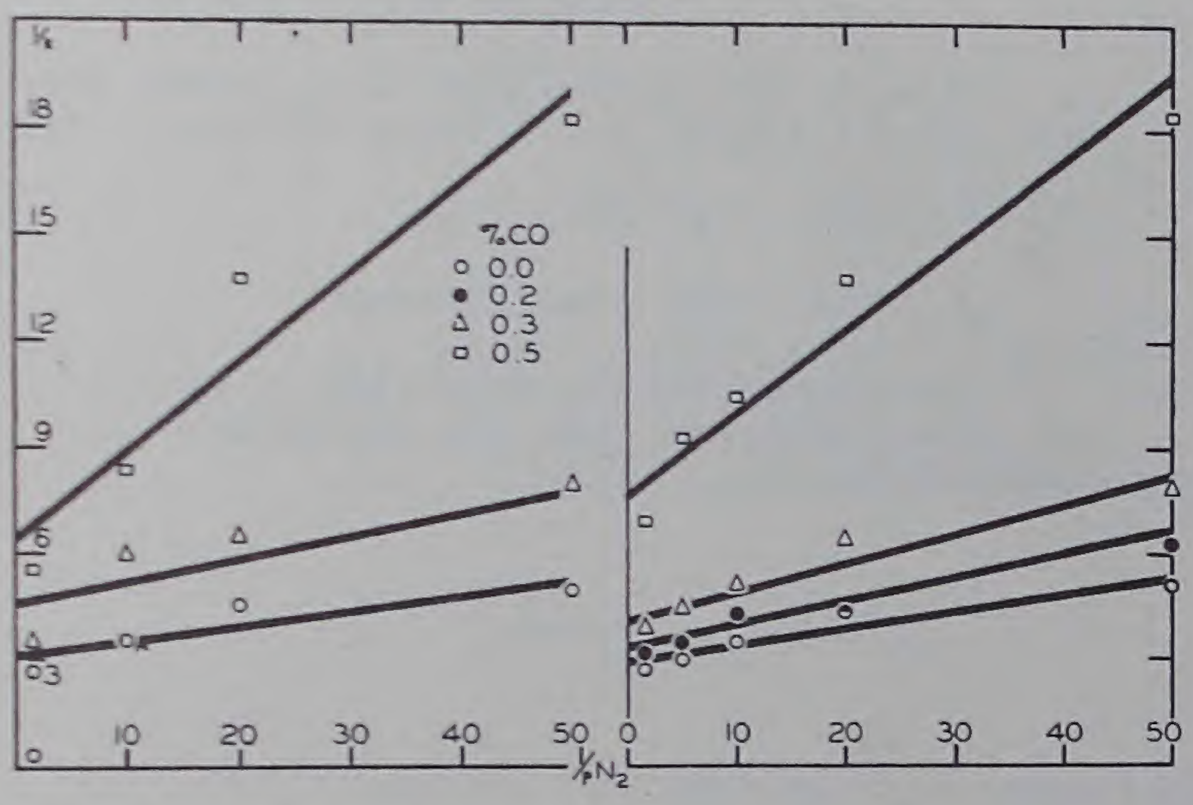


Figure 4

Non-competitive inhibition of nitrogen fixation in Azotobacter by carbon monoxide. (Ebersole, Guttentag and Wilson, 1944). Plotted according to equation 15.

Both slope and intercept change in the " double reciprocal" plot when inhibition is non-competitive.

If more than one molecule of inhibitor combines with one molecule of enzyme, the curve will be concave upward instead of linear. The appropriate form of equation (16) is then

$$(17) \quad v/v_i = 1 + (I)^r/K_i$$

$$(18) \quad \log (v/v_i - 1) = \log 1/K_i + r \log (I)$$

The number of molecules of inhibitor, r, combining with one molecule of enzyme is determined by estimating the slope of the line obtained when $\log (v/v_i - 1)$ is plotted against log (I). This type is illustrated in Figure 5, an example in which r equals 2 (Ebersole, Guttentag and Wilson, 1944).

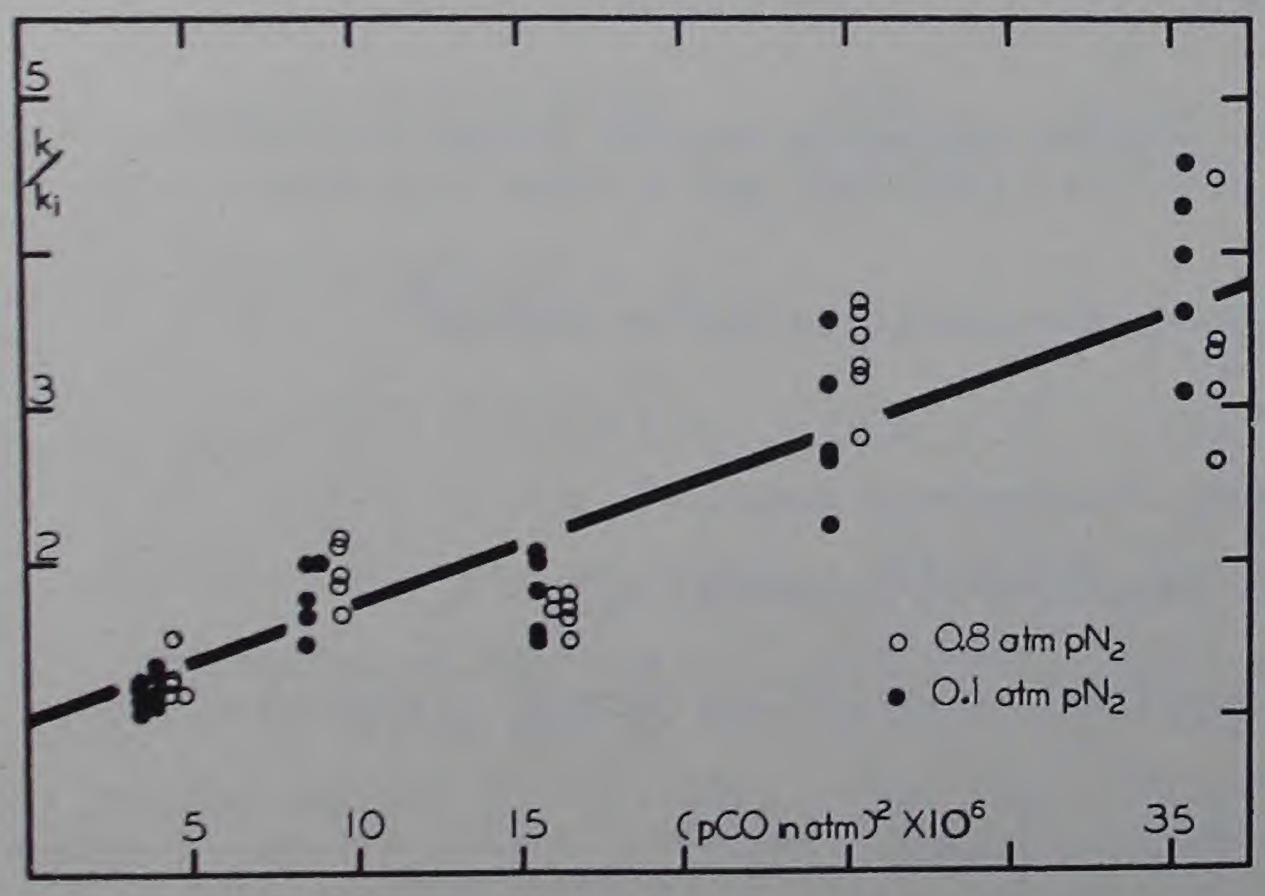


Figure 5

Non-competitive inhibition of nitrogen fixation in Azotobacter by carbon monoxide.

Plotted according to equation 17 with r = 2.

The points fall along the same line independent of concentration of substrate (pN_2).

CRITICISM, CORRECTION, MODIFICATION AND EXTENSION OF THE THEORIES

Although the mathematical formulations of section I have proved useful tools for examining proposed mechanisms of enzyme action and for purely descriptive purposes, such successes by no means establish their validity. When examined critically and tested with diverse types of enzymatic reactions, it is evident that they represent primarily first approximations applicable to a limited number of reactions. They are a convenient starting point for kinetic analysis, but the basic assumptions are too simplified to correspond to the many types of complex reactions catalyzed by enzymes. In this section we consider some of the criticisms that have been directed toward the elementary treatment given in section I and briefly discuss proposed modifications and extensions.

TEMPERATURE

The treatment of biological temperature coefficients in section I provides a satisfactory explanation of the commonly observed values of Q_{10} and has a certain logical appeal, but it is doubtful if the relatively simple equation of Arrhenius depicts accurately the influence of temperature on such complex phenomena as growth and respiration. Many writers (Buchanan and Fulmer, 1930; Belehradek, 1935) object to the indiscriminate use of this equation for description of biological processes and have emphasized especially the danger of attaching any theoretical significance to values of μ. The chief source of dissatisfaction with the equation is that often μ is not a constant but is a function of the temperature - that is, a plot of $\underline{\log}\ \underline{k}$ against $1/T$ yields a curve rather than a straight line. Buchanan and Fulmer (1930) argue that frequently a linear relationship between $\underline{\log}\ \underline{k}$ and $1/T$ depends on the method of plotting the data. If too small a unit is used for $\underline{\log}\ \underline{k}$, continuous variations of this quantity with $1/T$ will be masked, and an apparent straight line will be obtained.[1] Crozier and others have suggested as a possible explanation of the non-linearity that a biological process such as respiration, fermentation or growth is the resultant of a series of "catenary reactions". Different members of the series become the slowest at various temperatures, and this limits the velocity of the over-all reaction. If the reactions of the chain have different temperature characteristics (μ values) then the value of μ for the over-all process will change abruptly at definite "critical" temperatures. Booij and Wolvekamp (1945), among others, criticize this explanation. The mechanism based on the theory of absolute reaction rates to be discussed in the following section provides for a change in the slope of the line, though probably not as marked as is sometimes observed.

Some workers interpret temperature coefficients on a biophysical rather than a biochemical basis. The biophysical view holds that a biological temperature coefficient is a reflection of the influence of temperature on such physical processes as diffusion and viscosity rather than on a chemical reaction (Booij and Wolvekamp, 1945). An excellent discussion of the two points of view is given in the monograph by Belehradek (1935).

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[1] It should also be noted that many biologists determine μ graphically by merely drawing the line which appears to fit best the experimental points. Since such a procedure gives full scope for personal bias, its use is decidedly open to question. By well-established statistical methods the "best" straight line may be readily determined ("best" in the least square sense). This line will provide not only the best estimate of μ but also the standard error attached to this estimate. Accurate comparison of whether two values of μ differ significantly from each other is then possible (Eisenhart and Wilson, 1943). Bodansky (1937) has shown that a variable μ may result from selection of the wrong measure of reaction velocity. This highly important point of employing the proper function for measure of the velocity of a biochemical reaction when a proposed generalization is tested has been almost entirely overlooked in development of enzyme theory. This neglect may explain in part the confusion and contradictions encountered in this field.

CONCENTRATION OF SUBSTRATE

The fact that experimental observations are in harmony with the theoretical ones described by the Michaelis-Menten equation does not, of course, prove that the basic assumptions of their hypothesis are justified. Agreement of observation with theory indicates merely that the idea of the formation and decomposition of an unstable intermediate is a permissible hypothesis, not a necessary one. For example, the same sort of data would result if the union of substrate with enzyme is physical rather than chemical since equation (10) is formally identical with Langmuir's adsorption isotherm, $q = ap/(b + p)$, in which q is the quantity of gas adsorbed at pressure, p; a is a constant representing the quantity of gas adsorbed at saturating pressures; and b is a constant analogous to K_s. Agreement of the experimental data with either equation does not tell, therefore, whether the enzyme-substrate equilibrium is homogeneous or heterogeneous. This point will be referred to further in the next section when Medwedew's views are discussed.

Haldane (1930) and Lineweaver and Burk (1934) have extended the simple case treated by Michaelis and Menten to more complicated ones in which some of the restrictive assumptions have been removed. Mathematical derivations for these more complex instances have been provided, those of Lineweaver and Burk being more readily applicable as they are in linear form. As an example of such extensions, the objection of Briggs and Haldane (1925) will be cited since it emphasizes the important fact that the observed value of K_s (likewise K_1) need not represent a true dissociation constant and that the interpretation of the constant as such should be made with reserve. On the basis of molecular statistics, these workers rejected the assumption that the velocity of formation and dissociation of ES is infinite in comparison with the observed velocity of hydrolysis and present an alternative derivation:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + F + G$$

where k_1, k_2 and k_3 represent the velocity constants of the reaction indicated.

Using the same symbols as before, it is evident that the rate of formation of

$$ES \text{ is } \frac{d(ES)}{dt} = k_1 \left[(E) - (ES)\right]\left[(S)\right],$$

i.e., is proportional to the concentration of free enzyme and substrate.

The rate of decomposition of ES is $\frac{-d(ES)}{dt} = -k_2(ES) - k_3(ES)$; hence, the overall change in concentration of ES is:

$$(19) \quad \frac{d(ES)}{dt} = k_1\left[(E) - (ES)\right]\left[(S)\right] - k_2(ES) - k_3(ES)$$

As long as the rate of reaction, v, is constant then (ES) must be constant and hence $\frac{d(ES)}{dt} = 0$. Substituting this in equation (19) and solving for (ES),

$$(20) \quad (ES) = \frac{k_1 (E)(S)}{k_1(S) + k_2 + k_3} \quad \text{or}$$

$$(21) \quad (ES) = \frac{(E)(S)}{(S) + \frac{k_2 + k_3}{k_1}}$$

Clearly, if $\frac{k_2 + k_3}{k_1}$ is taken equal to Ks, equation (21) reduces to equation (9) of the Michaelis-Menten derivation. The remainder of the derivation of this modification is similar to that already given for the Michaelis-Menten equation. The derivation of Briggs and Haldane makes it evident that K_s calculated from experimental data may not be a true dissociation constant; for many purposes, however, it may be used as such.

ACTION OF AN INHIBITOR

Analysis of the action of different types of inhibitors has been important not only for enzyme theory but for the understanding of the pharmocology of drug action (see the excellent discussion of this point in McElroy, 1947). Because of this practical application it is not surprising that this phase of enzyme mechanism has been most thoroughly explored. In the previous section it was indicated that inhibitors of enzyme reactions fell into two general classes:

1. Competitive - the inhibitor competes with substrate for active groups on the enzyme, e.g., metabolic analogues of the succinic acid-malonic acid type. An important example of this type is inhibition by products of the reaction. Frantz and Stephenson (1947), in an interesting paper dealing with the hydrolysis of peptides, have recently extended the original analysis of Michaelis-Menten.

2. Non-competitive - the inhibitor combines with the enzyme independent of the presence of substrate, e.g., heavy metals with -SH groups.

Closer study reveals that this classification is an oversimplification and that other types of inhibition can occur. First, it should be noted that competitive and non-competitive inhibition are not restricted to the cited mechanisms. Either type may involve combination with prosthetic group rather than apoenzyme (McElroy, 1947). Moreover, other types of inhibition have been observed: Winzler (1943) reports, for example, that azide combines with the oxidized form of the Atmungsferment (ES) but not the reduced form (E). If the inhibitor combines only with ES but not with E, the velocity kinetic equation is:

$$(22)\quad 1/v_i = \left[1 + \frac{(I)}{K_{esi}}\right] 1/V^* + \frac{K_s}{V^*}\left[1/(S)\right]$$

("uncompetitive" inhibition, Ebersole, Guttentag and Wilson, 1944). The double reciprocal plot results in an unchanged slope but the intercept is increased by $[1 + (I)/K_{esi}]$. Examples, intermediate with respect to competitive and non-competitive inhibition, would arise if the affinity of the inhibitor for E and ES differ, i.e., $K_i \neq K_{esi}$, so that the slope and intercept of the double reciprical plot change by different factors.

A type of inhibition for which Burk has suggested the name "quadratic" or "discontinuous" should obtain when the process under measurement is a series of reactions with the following characteristics:

$$E + S \rightleftarrows ES \xrightarrow{\text{slow}} P \xrightarrow{\text{fast}} B \longrightarrow C$$

C is a compound that is eventually required in a reaction to make ES available; the inhibitor combines reversibly with C so that (C)/(C total) is equal to $1/[1 + (I)/K_i]$. The kinetic equation is quadratic with components S, I, E, ES, C and CI; the double reciprocal plot forms lines with different intercepts and initially, near zero slopes; the slopes eventually change to a common slope equal to that obtained when (I) is zero. Experimental realization of this type has been described by Lineweaver and Burk (1934) for diffusion of carbon dioxide in photosynthesis; by Commoner (1939) for inhibition of respiration of yeast by cyanide at various concentrations of glucose; and by Winzler (1943) for respiration of yeast on different substrates and inhibition by cyanide as a function of the partial pressure of oxygen.

THE GENERALIZED THEORY OF STRAUS AND GOLDSTEIN

A chief source of the criticisms of the Michaelis-Menten treatment is its assumption of certain properties for the system that may not always obtain. For example, it is assumed that the reaction is pseudomonomolecular instead of bimolecular as is indicated by the formulation. Straus and Goldstein (1943) and later Goldstein (1944) derived the velocity equations rigidly so that any simplifying assumptions could be introduced only after they were shown to be valid for a particular system. It is instructive to compare their generalized derivations with the more specific ones already discussed.

Substrate alone

If E is total enzyme, E_f is free enzyme, S, total substrate and S_f, free substrate, then the correct velocity equation can be derived:

$$E + S \longleftrightarrow ES$$

$$K_s = \frac{(E_f)(S_f)}{(ES)}$$

$$= \frac{E(1-a)(S-aE)}{aE}$$

$$E = E_f + ES$$

$$S = S_f + ES$$

Let a, fractional activity be defined:

$$a = \frac{ES}{e} = \frac{v}{V_m}$$

$$aE = ES$$

$$E_f = E(1-a)$$

$$S_f = S - aE$$

$$(23) \quad S = K_s \frac{a}{1-a} + aE$$

A noteworthy contribution of Straus and Goldstein was to emphasize that it cannot always be assumed that (E) is negligible since the important consideration is not the absolute concentration of E but its concentration with reference to K_s. Accordingly, they expressed (E) and like quantities in specific concentrations, $E' = E/K$, which, analogous to specific gravities, are dimensionless. Equation (23) is put in the specific concentration form if divided by K_s:

$$(24) \quad S' = a/(1 - a) + aE'$$

This is the complete equation, but, if E' is very small, as is usually true, the last term is negligible, so it reduces to the simple Michaelis-Menten form. The question of when can this be done is best answered after consideration of inhibition.

Non-competitive inhibition

Non-competitive inhibition is entirely analogous to the substrate-enzyme equilibrium since the significant reaction is $E + I \longleftrightarrow EI$. It is assumed that concentration of substrate is high enough to saturate the free enzyme; consequently a, the fractional activity, is equal to E_f/E. From the equilibrium

$$K_i = \frac{(E_f)\ (I_f)}{(EI)} = \frac{(E_f)(I - EI)}{(EI)}$$

the complete equation (25B) is derived as before:

$$(25B) \quad I' = (1 - a)/a + (1 - a)\ E'_i$$

Since $(1 - a)\ E'_1$ is equal to $(EI)'_i$, the specific concentration of combined inhibitor, the first term in (25B) must represent the free inhibitor. Non-competitive inhibition therefore is divided into three zones according to how many terms of equation (25B) it is necessary to use: In zone A essentially all inhibitor is free so that

$$(25A) \quad I' = (1 - a)/a;$$ in zone C the inhibitor is all combined so that

$$(25C) \quad I' = (1 - a)\ E'_i$$

In zone B the complete equation must be employed to describe the behavior of the enzyme-inhibitor system. The boundaries of E' are established by a graphical method based on the choice of a permissible error in a. For a = 0.01, the boundaries are approximately: Zone A, $E' < 0.1$; Zone C, $E' > 100$ (See Figure 1 in Straus and Goldstein, 1943).

Competitive inhibition

When the inhibition is competitive, two equilibria must be satisfied

$$(I)\quad \frac{(E_f)(I_f)}{(EI)} = K_i \qquad\qquad (II)\quad \frac{(E_f)(S_f)}{(ES)} = K_s$$

The following relationships are easily verifiable by consideration of these two equilibria:

$E = ES + EI + E_f$ $\qquad (EI + E_f)/E = (1 - a)$ or $EI = (1 - a)E - E_f$

in which $a = ES/e$.

Solving (II) for E_f and substituting in (I), the following complicated expression results:

$$(26\ B_i\ B_s)\quad \underset{\text{Total}}{I'} = \underset{\text{Free}}{\left[(S' - aE'_s)\left(\frac{1-a}{a}\right) - 1\right]} + \underset{\text{Combined}}{\left[1 - a\left(1 + \frac{1}{S'-aE'_s}\right)\right]E'_i}$$

The letters after the equation number signify that both inhibitor and substrate are in zone B; i.e., this is the most general and rigid form of the equation. Simplification is introduced by neglect of E_f which is rather small when both EI and ES are present:

$$(27\ B_iB_s)\quad \underset{\text{Total}}{I'} = \underset{\text{Free}}{\left[S' - aE'_s\right]\left[(1 - a)/a\right]} + \underset{\text{Combined}}{(1 - a)E'_i}$$

The fact that in general enzyme systems will operate in zone A with respect to S but some other zone with respect to I allows the writing of the following simplified useful variants of $(27\ B_iB_s)$:

$$(27\ A_iB_s)\qquad I' = (S' - aE'_s)\left[(1 - a)/a\right]$$

$$(27\ B_iA_s)\qquad I' = S'\left[(1 - a)/a\right] + (1 - a)E'_i$$

$$(27\ A_iA_s)\qquad I' = S'\left[(1 - a)/a\right]$$

Comparison of equation $(27\ B_iA_s)$ with (25B) (the corresponding one for non-competitive inhibition since the system is in zone A with respect to substrate) reveals that they are identical in form except for the multiplier, S'. The corresponding zones for competitive inhibition are accordingly:

Zone A $\quad I' = S'(1 - a)/a \qquad$ when $E'_i/S' < 0.1$

Zone C $\quad I' = (1 - a)E'_i \qquad$ when $E'_i/S' > 100$

One series of "dose-effect" curves will suffice for both competitive and non-competitive inhibition since the corresponding equations differ only by the appropriate multiplier. These curves are prepared by plotting a against log I' for different values of E'_i. Such curves have the following characteristics: (1) all curves for E'_i (non-competitive) or E'_iS' (competitive) equal to or less than 0.1 are represented by a single symmetrical curve with inflection at $a = 0.5$ and a slope of 0.575 at that point; (2) curves for E'_i and (E'_iS') greater than 0.1 become progressively steeper and spaced out until at zone C they are parallel and essentially logarithmic curves with slope, 1.15 (See Figures 2 in Straus and Goldstein, 1943 and Goldstein, 1944).

From an analysis of observed "dose-effect" curves Straus and Goldstein deduced many important properties of the enzyme system: cholinesterase-physostigmine-acetylcholine. They emphasize, however, that such analysis is not restricted to this particular system. Among the specific and general properties revealed by the analysis were:

1. Most enzyme systems fall in zone A as the dissociation constants usually exceed 10^{-5}; (E) would have to exceed 10^{-4} then to be in zones B or C. In vivo or even in tissue cultures the effective concentration of E may be sufficiently high with systems having low values of K to land in B or C. It is of interest that competitive inhibition cannot exist in zone C.

2. From the slope and other properties of the "dose-effect" curve the number of molecules, n, of inhibitor or substrate combining with enzyme can be determined.

3. In zones A and B dilution of enzyme-substrate-inhibitor leads to a decrease in inhibition beyond that arising from mere dilution. This effect may cause errors of interpretation when studying enzyme systems taken from body fluids and diluted for measurements.

4. Inhibition depends markedly on the concentration of substrate; effects deduced from studies in vitro in which excess substrate is added may be quite different from effects in vivo where substrate concentration may be much lower.

5. Studies on the kinetics of the displacement of I by S and S by I allow calculation of the velocity constants of the reactions

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad\qquad E + S \underset{k_4}{\overset{k_3}{\rightleftharpoons}} ES$$

For the cholinesterase system these were:

k_1 (combination) $= 8.3 \times 10^5$ $\qquad$ k_2 (dissociation) $= 0.026$

$K_i = 3.1 \times 10^{-8}$ $\qquad$ $K_s = 1.25 \times 10^{-3}$

k_3 (combination) $= 260$ $\qquad$ k_4 (dissociation) $= 0.32$

$\overline{E}$ could not be definitely evaluated but was less than 1.8×10^{-8} (diluted serum); $\overline{E'}_i < 0.58$ and is in or near zone A.

The original papers should be consulted for the wealth of theoretical and experimental detail on these and other properties revealed by their analysis. It is evident, however, from this brief summary that the authors have succeeded well in establishing the point made in their initial paper:

> "The classical treatment of the kinetics of enzyme reactions has been based upon the assumption of a very small concentration of enzyme centers acting according to the laws governing first order reactions (pseudomonomolecular). In this paper we shall show that under a number of common conditions such treatment cannot adequately describe the behavior of the system but that a more complete analysis must be employed. Enzyme-inhibitor and enzyme-substrate systems will be shown to behave in three distinct ways depending upon the concentrations of the reactants and the dissociation constant of the system. The boundaries of these three "zones of behavior" will be established on a kinetic basis applicable to all such systems, and the qualitative and quantitative differences in behavior will be demonstrated."

PROPOSED MECHANISMS FOR ENZYME REACTIONS

The approach discussed in the preceding section has been only indirectly concerned with the mechanism of the action of enzymes. For convenience, the formation of a rather vaguely defined unstable intermediate is suggested, but the major goal has been the development of equations that describe the rate of the reactions. In this section we shall consider the efforts that have been made to apply to enzyme reactions modern physical-

chemical theory that seeks to "explain" or predict the rate of a chemical reaction on the basis of the physical-chemical properties of the molecules involved. Although little success has yet been had with even the relatively simple systems of the chemist, application to biological reactions does not appear to be hopeless. Such efforts are justified if for no other reason than that: (a) they emphasize that agreement with a given equation derived on the basis of a particular mechanism does not constitute proof of that mechanism; (b) they furnish some suggestions about the properties of the transient intermediates that are implicit in all the proposed schemes thus clothing these with some semblance of reality.[1]

COLLISION THEORY

As has been already mentioned, Arrhenius initially proposed the empirical relation, $\ln k = B - A/T$; later the equation was derived from theoretical considerations. The modern form of the equation is: $d \ln k/dT = E/RT$, where $\underline{E}$ is the "energy of activation" of the molecule undergoing reaction, $\underline{k}$ is the measured velocity constant of the reaction and the other symbols have their usual significance. On integration, this equation becomes $k = Se^{-E/RT}$.

The next step in the development of the theory was to determine the significance of the constant, $\underline{E}$, and to provide methods for its calculation from measurable properties of the molecules. For unimolecular reactions, S may be regarded as a frequency and is approximately constant (order of 10^{13}). For bimolecular reactions (most important for enzyme reactions) $\underline{S}$ is regarded as the collision frequency. In many reactions straight-forward calculations based on the kinetic theory of gases provide satisfactory results. These reactions include both those that occur in the gaseous phase and in solution (Daniels, 1943).

In other reactions the discrepancy between the observed and calculated values is well beyond experimental errors. Moelwyn-Hughes (1932) has discussed these and has suggested possible explanations for the discrepancies. He concluded, however, that none of the suggestions appeared to be satisfactory. Hinshelwood (1926) and Jowett (1929), among others, have provided corrections to take care of factors not considered in the original treatment. Hinshelwood's correction deals with the energy of activation; he suggests that part of the energy required may be used to satisfy the so-called internal degrees of freedom of the molecule. These degrees of freedom may be roughly considered as arising from the ability of the molecules or groups in the molecules to orient themselves in different spatial arrangements. Essentially, Hinshelwood's concept is that the energy of activation as determined by the change in $\ln \underline{k}$ with temperature is not the true energy of activation but must be corrected according to the following equation:

(28) $k = Z\, e^{-E/RT}(E/RT)^F(1/F)$ in which $\underline{Z}$ represents the "collision term" and $\underline{F}$ is equal to the internal degrees of freedom of the molecule and may be evaluated from the following relation:

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[1] See, for example, the recent review of LuValle and Goddard (1948) dealing with the mechanism of enzymatic oxidations and reductions which appeared after this manuscript had been completed. In addition to discussing many aspects of enzyme mechanisms that are the subjects of this section, these authors propose a general mechanism applicable to many classes of oxidation-reduction enzymes including oxidases, peroxidases, catalase and dehydrogenase. The mechanism, based in part on the views recently advanced by Pauling and by Michaelis, has two basic assumptions: (1) All reactions proceed through a series of univalent electron changes; (2) A ternary complex of donor, enzyme and acceptor must be formed for the reaction to go to completion. Oxidation is believed to proceed via semiquinones that are formed and remain within the enzyme complex. Such a mechanism, they state, avoids chain reactions and non-specific oxidations and thus allows for enzyme specificity. The authors develop general equations for the reaction kinetics. They claim the following advantages for the scheme: (1) it reduces all enzymic oxidation-reductions to a basic mechanism; (2) it permits enzymes to retain specificity while producing semiquinones and radicals; (3) it explains oxidase reactions without oxygen activation.

(29) E (observed) + RTF = E (true), which leads to the equation:

$$(30)\quad F = \frac{E_1 - E_2}{R\,(T_1 - T_2)}$$

Further details regarding the collision theory and chemical kinetics can be obtained from the following references: Symposium on Kinetics of Reaction - Chem. Rev. 17: 43-136, 1935; Moelwyn-Hughes (1933a) and Hinshelwood (1934).

Moelwyn-Hughes (1933) who has made important contributions to the theory as well as to the experimental basis of chemical kinetics, has applied modern theories of reaction kinetics to enzyme reactions. A few examples from his initial paper will suffice to illustrate the type of problem undertaken.

Kinetics of inactivation of enzymes

At optimum pH it is assumed that inactivation of enzyme is due primarily to collision with the solvent molecules (water).[1] The collision term, Z, may be calculated by two methods. First, an extension of Einstein's treatment of Brownian movement will give the frequency of collision between the large solute (enzyme) and small solvent molecules. When the proper calculations, which involve the molecular diameter of the enzyme and the viscosity of water, are made, a value of 9.1×10^{14} for Z is obtained. A second method for determining Z is an application of the kinetic theory of gases; the enzyme molecule is regarded as a wall against which the solvent molecules collide as do gas molecules in a container. This method yielded a value of 4.1×10^{14} for Z. Since good checks were obtained by the two methods, the collision term may be taken as approximately 5×10^{14}.

If this value is substituted in Hinshelwood's equation (28), the expression, $k = (5 \times 10^{14})\, e^{-E/RT}\, (E/RT) \times 1/F$ is obtained. For enzymes, F has not been estimated directly, but values of F may be selected so that k calculated from the equation will agree with the experimentally determined values. Moelwyn-Hughes (1933) has made the necessary calculations for a number of proteolytic enzymes whose E values are known. He found that if the following values of F were assumed, the calculated k's agreed with the observed velocity constants: trypsin-kinase, 12; pancreatic proteinase, 9; and enterokinase, 16. These necessary values appear to be reasonable and compare favorably with one another. It was concluded that the rate of inactivation by solvent molecules is such that it can be satisfactorily accounted for on the basis of collision activity alone.

Energetics of enzyme reactions

In general, the extended theory of Hinshelwood appears to be quite satisfactory in accounting for some aspects of enzyme kinetics, but several objections may be raised against it. Chief of these is that at times calculation of the so-called energy of activation by Hinshelwood's equation (29) leads to estimates which do not appear to be consistent with modern concepts of catalysis. For example, the figures which must be assumed for F in several instances lead to calculated values of the true energy of activation which are several times larger than would be expected. Thus for hydrolysis of sucrose the calculated F is 52 and E, 39,000 cal. Since saccharase is superior to the H-ion as a catalyst, the reaction catalyzed by it should have a lower critical increment; however, E for hydrolysis of sucrose by acids is only 25,600 cal.

In the decomposition of hydrogen peroxide by liver catalase, F is apparently 170 which corresponds to an E of 101,800 cal. The same reaction catalyzed by the iodide ion has a critical increment of 13,500 cal. The calculated F for hydrolysis of starch is 121, and for action of pepsin on edestin is 64. In addition to the argument based on abnormal values for the critical increments, these estimates of F appear to be much too large. If 10 to 15 internal degrees of freedom contribute to the energy of activation when enzymes

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[1] The effect of H^+ and OH^- ions on the inactivation is also discussed by Moelwyn-Hughes.

are thermally destroyed, estimates of 50 to 100 for the numbers involved when a substrate molecule is decomposed on a portion of the enzyme surface are not plausible. Moelwyn-Hughes concludes,

> "The idea that a large number of internal degrees of freedom usually contributes to the energy of activation in reactions catalyzed by enzymes does not now appear as attractive as it did a few years ago. As far as the information dealt with in the present inquiry entitles us to decide, the theory is not only untenable but unnecessary. Despite much uncertainty connected with finding the true value for the energy of activation, it is probable that the apparent critical increment is much nearer the mark than the large corrected values given above."

Although the extended theory of Hinshelwood might explain thermal inactivation of enzymes, it does not appear to be involved in the mechanism of true enzymatic catalysis - at least as far as our present information goes. More recent data discussed by Moelwyn-Hughes (1937) suggest that even the absolute velocity of inactivation of enzyme may be accounted for by a simple collision theory.

CHAIN REACTION THEORY

Physical-chemical investigations of several photochemical reactions revealed the anomalous situation that the quantum efficiency was greater than unity; in some examples extremely large values were encountered. To explain these surprising results, Bodenstein in 1913 proposed the chain theory of chemical reactions, the details of which are given in the review of Bodenstein (1930), the monograph of Semenoff (1935), or any modern text of physical chemistry.

Moelwyn-Hughes (1937) emphasized that many of the characteristics of enzyme-catalyzed reactions are those usually associated with chain reactions. Among these are:

1. The velocity of a chain reaction is a function of the number of active centers generated per unit time in unit space and of the average length of chain. For this reason there is, in general, no simple algebraic relation between the instantaneous rate of reaction and the concentration of reactants.

2. A chain may be abbreviated by the presence of extraneous substances which do not undergo chemical change themselves.

3. The chains may require time to develop so that a period of induction is observed.

Some of the properties of chain reactions are not exhibited by enzyme reactions, such as the rate of reaction depending on the dimensions of the container, or the occurrence of explosions because of branching of the chains. More or less plausible explanations have been suggested to account for such discrepancies. Since the prime requisite of a chain reaction is the occurrence of one highly exothermic step, the natural reactions to be considered in this light will be those catalyzed by the oxidative type of enzyme (oxidases, peroxidases and dehydrogenases). As has been already indicated, many of the hydrolytic types appear to be accounted for on the basis of collision theory.

General solution of the effect of substrate concentration on reaction velocity

Although the equation of Michaelis and Menten is adequate for many reactions, it does not always describe the experimental findings, e.g., those of the xanthine oxidase system. Moelwyn-Hughes (1937) supplied a more general derivation, based on considerations of possible chain reactions, which he claimed more closely fits all the facts. This derivation is of interest as it illustrates how formally equivalent kinetic equations result even though based on quite different assumed mechanisms.

Consider the following reactions occurring in the chain:

1. $E + S \longrightarrow X_o$ — Chain initiator
2. $X_o \longrightarrow P + X_n$ — Chain Propagators
3. $X_n + S \longrightarrow X_o$ — Chain Propagators
4. $X_o + S \longrightarrow E + 2S$ — Chain Breaker

(S) - Conc. Substrate
(E) - " Enzyme
(P) - Products of reaction.
(X_o), (X_n) - ions, molecules and radicals of unspecified composition which appear and disappear in the chain.

The rate of formation of X_o is

$$(31) \quad dX_o/dt = Z_1 (E)(S) + Z_3 (X_n) (S) \alpha$$

where α denotes a term to take account of fact that chains may be branched. The rate of decomposition of X_o is,

$$(32) \quad - dX_o/dt = -Z_2(X_o) - Z_4(Z_o) (S).$$

Therefore,

$$(33) \quad \frac{dX_o}{dt} = Z_1 (E) (S) + Z_3 \alpha (X_n) (S) - Z_2 (X_o) - Z_4 (X_o) (S)$$

Similarly,

$$(34) \quad dX_n/dt = Z_2(X_o) - Z_3 (X_n) (S)$$

The Z terms are unknown functions dependent on size of molecule, temperature, etc. but <u>independent of substrate concentration</u>.

When the chains have established themselves, both dX_o/dt and dX_n/dt will equal zero (no change in concentration at equilibrium); hence,

$$(35) \quad Z_1(E) (S) + Z_3 \alpha (X_n) (S) = Z_2(X_o) + Z_4 (X_o) (S) \text{ from (33)}$$

$$(36) \quad \text{and} \quad Z_2 (X_o) = Z_3 (X_n) (S) \quad \text{from (34)}$$
$$(X_o) = Z_3 (X_n) (S)/Z_2$$

Substituting the values from (36) into (35),

$$(37) \quad Z_1(E) (S) + Z_3 \alpha (X_n) (S) = Z_3(X_n) (S) + \frac{Z_3 Z_4}{Z_2}(X_n) (S)^2$$

$$(38) \quad Z_3(X_n) (S) = \frac{Z_1(E) (S)}{(1 \cdot \alpha) + \frac{Z_4(S)}{Z_2}}$$

Now the velocity of the reaction is $v = dP/dt = Z_2(X_o) = Z_3(X_n) (S)$ from (36); and from (38),

$$(39) \quad v = \frac{Z_1(E) (S)}{(1- \alpha) + \frac{Z_4}{Z_2} (S)} = \frac{Z_1 Z_2 (E)/Z_4}{1 + \frac{Z_2}{Z_4 (S)} (1-\alpha)}$$

It is evident that if $(1-\alpha) \gg \frac{Z_4}{Z_2} (S)$, then $v = \frac{Z_1(E) (S)}{(1- \alpha)}$, that is, the reaction is nearly unimolecular. Since the unimolecular constant is

$$k = 1/S \cdot d(P)/dt = \frac{1}{S} \cdot v = \frac{Z_1(E)}{(1- \alpha)},$$

$\frac{1}{(1-\alpha)}$ may be regarded as the average number of links in the chain, and $Z_1(E)$ (S) as number of initial centers generated per unit volume in unit time. On the other hand, if $(1-\alpha) \ll \frac{Z_4}{Z_2}$ (S), then $v = \frac{Z_1 Z_2}{Z_4}(E)$, and the reaction becomes zero order. These two conditions describe enzyme reactions which have a substrate-velocity curve similar to that shown in Figure 1.

At low values of (S) the rate of reaction is almost directly proportional to concentration of substrate (unimolecular reaction); at high values of (S) the rate is practically independent of this variable (zero order reaction). This transition in order of the reaction is comparable with that found in gas reactions in which the order is bimolecular at low pressures and monomolecular at high. The unusual type of substrate-velocity curve encountered in the xanthine oxidase system will be discussed in a succeeding section.

Finally, an identification of Ks with this generalized equation (39) based on a postulated chain reaction should be made. According to equation

(10') $Ks = (S)\ (V/v - 1)$. To find the value of the maximum velocity, V, equation (39) is differentiated with respect to (S) and set equal to zero. The maximum velocity will be reached when α is equal to unity and will be:

$$(40)\quad V = \frac{Z_1 Z_2}{Z_4}(E)$$

When this value and that for $\underline{v}$ are substituted into equation (10') equation (41) results:

$$(41)\quad Ks = \frac{Z_2}{Z_4}\ (1 - \alpha),$$ which states that the Michaelis constant is inversely proportional to the average length of the chain.

Moelwyn-Hughes (1937) is of the opinion that, considering the values of Ks for hydrolytic reactions (ca. 10^{-2}) in comparison with those for oxidative reaction (ca. 10^{-6}), chain reactions are important for the latter only.

The luciferin-luciferase system

This system is of particular interest because light is emitted through oxidation, the reverse of photochemical reactions initiated by light. The Michaelis constant, calculated from equation (10), turns out to be negative which, of course, can have no physical meaning. However, the influence of substrate concentration on velocity may be fully described by equation (39) provided $(1 - \alpha)$ has a negative value. There is evidence that luminescence occurs at the termination of a chain. The chains appear to be fairly short, and the number of links corresponds roughly to the number of oxygen molecules which disappear per quantum of light emitted. Several other properties of this system which do not receive easy explanation by application of ordinary collision mechanisms are resolved by treating the reaction as a chain type. (Moelwyn-Hughes, 1937).

The xanthine oxidase system

The substrate-velocity curve at low concentrations of substrate for this system is quite different from that usually encountered in that the curve has abrupt discontinuities. The collision hypothesis does not appear to be adequate, but application of a chain mechanism provides a formal explanation for the various portions of the curve (Moelwyn-Hughes, 1937). For example, above a certain concentration of substrate the velocity decreases with increase in substrate concentration as was also noted in the luciferase system. In terms of the simple Michaelis-Menten theory, this means that Ks is negative. However, in terms of the chain theory, it merely indicates that α is greater than unity, i.e., that the chains are branched, and the system has explosive tendencies. Although recourse to a chain mechanism may provide a convenient and even a logically satisfactory explanation of observations, convenience and satisfaction should not be mistaken for scientific proof. The unusual behavior of the xanthine oxidase system may originate from some particular property of the enzyme preparation, the technique used in the measurements, or the presence of an undetected interfering reaction.

Catalase-hydrogen peroxide system

Several workers, including Haber and Willstäter (1931), Richter (1932) and Weiss (1937), have postulated a chain mechanism for the decomposition of hydrogen peroxide by catalase. Haldane (1931, 1932) states, however, that the results with this system can be accounted for on a purely collisional basis and is of the opinion that the majority of biochemists will demand strong experimental proof that a chain mechanism operates in this and other enzymatic reactions. He advances the following objections against its acceptance:

1. The chain theory renders unintelligible the proportionality observed between enzyme concentration and velocity of reaction. The "theory" calls for a velocity constant which depends on the square root of the enzyme concentration, whereas the velocity actually observed is proportional to the first power.

2. The velocity should be materially reduced by impurities in the enzyme preparation since these would break the chains; such reduction is not observed.

3. The chain theory does not account for specificity of enzymes.

4. No mechanism is provided by the theory for transfer of the energy of oxidation reactions. In most biological oxidations the energy is transferred to another reaction; the transfer often involves the interaction of at least four molecules which must be held simultaneously at a specific surface. The occurrence of such a reaction in a homogeneous medium is difficult to visualize.

Although his calculations indicated that the observed rate of decomposition of hydrogen peroxide by catalase could be predicted from simple collision theory, Moelwyn-Hughes (1937) believes that the chain theory furnishes the most reasonable mechanism for the decomposition of H_2O_2 by catalase. His equation (39) states that the rate of reaction is proportional to the first power of the enzyme concentration, (E), which invalidates the first criticism of Haldane. Moelwyn-Hughes also suggests that the agreement between the "observed" $\underline{k}$ and that predicted by the collision hypothesis may be fortuitous in that the observed value is in error. He emphasizes that the conclusion that each activating collision brings about chemical change is based on the not too well founded assumptions that the iron in the enzyme preparation is entirely from catalase and that one atom of iron found by analysis corresponds to one molecule of the enzyme. He concludes:

> "Conformity with a chain theory of the reaction now requires that the actual quantity of enzyme present is less than the value hitherto estimated, in the same proportion as 1 is less than the number of links in the stable chain."

THE QUANTUM THEORY OF MEDWEDEW

The interpretation of the kinetics of enzyme reactions discussed in the foregoing sections has been based on the postulated formation of a more or less stable intermediate composed of substrate and enzyme. As has been indicated, formulation of enzyme reactions on this basis is in satisfactory agreement with most of the existing data concerned with the kinetics of such reactions. The intermediate hypothesis is not entirely adequate, however, and alternative explanations have been sought to account for the discrepancies between observations and theory. Medwedew, a Russian biophysicist, suggests that the intermediate-substrate-enzyme complex theory be discarded as it is out-of-date and inconsistent with modern physical theory. He claims (Medwedew, 1937) that the theories based on the concept of intermediate formation rest upon erroneous foundations for these reasons:

1. The time interval for reactions catalyzed by enzymes is much too small to allow for formation and decomposition of a stable intermediate. Calculations based on the hydrolysis of sucrose indicate that the duration of the hypothetical intermediate is of the order of 10^{-3} sec.; such a time interval, Medwedew states, is much smaller than the usual duration of a chemical compound.

2. If the reciprocal of the Michaelis constant represents the constant of affinity between substrate and enzyme, the values calculated on the basis of the Michaelis-Menten equation are far too high especially for hydrolytic reactions. Calculations of the heat of dissociation (or formation) of the intermediate based on the affinity constant gives values which are much larger than the observed heat of reaction. According to Medwedew, these two values should be closely related.

3. The magnitude of the temperature coefficient of a reaction should be closely related to the observed heat of the reaction since it is theoretically derived from the latter. The heats of reaction, however, are much too small for the observed temperature coefficients especially for hydrolytic reactions in which the energy change is very slight.

Derivation of statistical mechanical theory

Medwedew (1937) proposes to replace the intermediate complex theory with one based on statistical mechanics in which there is no formation of a stable compound between enzyme and substrate but only contact for energy exchange.[1] A brief formulation of this theory follows; for details of the concepts proposed by Medwedew the original publications should be consulted.

Denote substrate molecules by Sa, Sb, etc.; R and P are products of decomposition of the reaction; F, the enzyme; E, energy of activation; h_ν, quantum of energy.

1. $F + h_\nu \longleftrightarrow F^*$
2. $S + h_\nu \longleftrightarrow S^*$
3. $S^* \longrightarrow R^* + P$
4. $R^* \longrightarrow F^* + R$

An enzyme molecule, F, becomes activated (F^*) through taking on a quantum of energy; this energy is derived from that liberated in decomposition. The activated enzyme molecule collides with a molecule of substrate and through an "inelastic collision of the second order" transfers this quantum of energy to the molecule of substrate which in turn becomes activated; the enzyme molecule is of course deactivated in the exchange. Now two things may occur:

1. If the energy of the molecule of substrate is such that the following relation holds: $\epsilon Sa \geqslant E - h_\nu$ where ϵS_a denotes energy of molecule, the reaction occurs $F^* + Sa \longrightarrow F + S_a^*$ followed by $Sa^* \longrightarrow R^* + P$. R^* may now give up its energy to F to reactivate the enzyme or the latter may be reactivated by energy of reaction.

2. If, however, the energy of the molecule of substrate is such that $\epsilon_{Sb} < E - h_\nu$, then the following reaction occurs:

$F^* + S_b \longrightarrow F^* S_b$. This intermediate compound does not dissociate. The activated enzyme is tied up until by collision with some other activated substance, as R^*, it secures sufficient energy to bring about decomposition of S_b. The existence of this undissociated complex is made use of in the theory for explanation of inhibition of initial velocity by substrate (induction), inhibition by other substrates, and the influence of other factors such as pH and coenzymes on induction and inhibition.

Calculation of reaction rates

On the basis of the foregoing concepts Medwedew (1937) has derived an expression relating substrate concentration to velocity of reaction for replacement of the rejected Michaelis-Menten expression:

If (ø) is the concentration of active enzyme molecules which at a given time are engaged in transferring energy to the substrate, then the rate of change in (ø) with con-

[1] Compare with recent claims of Rothen (1947) regarding action of enzymes at a distance.

centration of substrate ($\frac{d\phi}{dS}$) will depend on both the total number of active enzyme molecule (F*) and (ϕ) or

(42) $d(\phi) / d(S) = B \left[(F^*) - (\phi)\right]$. B is a constant which is independent of time, concentration of (F*) and of (S). Integrating this expression and substituting the lower limits, (S) = 0 and (ϕ) = 0:

(43) $B = 1/(S) \ln \frac{(F^*)}{(F^*) - (\phi)}$

(44) $(\phi) = (F^*) \left[1 - e^{-B(S)}\right]$

If G is the probability that transfers from (ϕ) to substrate are effective then:

(45) $v = -d(S) / dt = G(\phi) = G(F^*) \left[1 - e^{-B(S)}\right]$. Integrating both sides of this equation between the limits, t = 0, (S) = a and t = t, (S) = (a-x):

(46) $k = \frac{1}{Bt} 2.303 \log \frac{1 - e^{Ba}}{1 - e^{B(a-x)}}$ in which the constant G (F*) in equation (45) has been replaced by k.

Substitution of the data originally obtained by Michaelis and Menten (1913) for test of their equation shows equally good agreement with expression (46) derived by Medwedew. This illustrates the point that agreement between observations and predictions based on some particular theory does not necessarily constitute proof of that theory. Other hypotheses may fit the data equally well.

Finally, the relation of the constant B in equation (42) to the Michaelis constant, Ks, should be indicated. The Michaelis constant is defined as substrate concentration at which velocity is equal to 1/2 V. Then, since

$v = G(\phi)$, when $v = 1/2\ V$
$G(\phi) = 1/2\ V$ or $(\phi) = \frac{1/2\ V}{G}$.

At substrate concentration, $S_{\frac{1}{2}}$, at which $v = 1/2\ V$, $S_{\frac{1}{2}} = K_s$ by definition. Also $(F^*) = V/G$, since the maximum velocity, V, will obviously be equal to G (F*). Substitution of these values in (43):

$$B = 1/S_{\frac{1}{2}} \ln \frac{V/G}{V/G - 1/2\ V/G} = 1/S_{\frac{1}{2}} \ln 2$$

$$S_{\frac{1}{2}} = 0.69/B = K_s$$

Activation energies and temperature coefficient of reaction

To obtain an energy of activation, E, which is satisfactory for calculation of the absolute velocity rates for certain reactions, it has been necessary to introduce correction factors such as those contained in the Hinshelwood's expression. In general, this particular treatment was unsatisfactory for enzyme reactions and has been largely rejected by the original proposer, Moelwyn-Hughes (1933; 1937). Medwedew (1937) has suggested the following scheme for determining the true activation energy; Let A be the apparent energy of activation calculated from data in the usual manner: since

(47) $A = \ln \frac{k_2}{k_1} \frac{R\ T_1 T_2}{T_2 - T_1}$ and $k = Z\ e^{-E/RT}$ (Z is collision term, see equation 28)

(48) then $\ln k_1 = \ln Z - E_1/RT_1$ and $\ln k_2 = \ln Z - E_2/RT_2$

(49) $\ln k_2 - \ln k_1 = E_1/RT_1 - E_2/RT_2$

(50) $\ln k_2/k_1 = \frac{E_1T_2 - E_2T_1}{R\,(T_1T_2)}$ or substituting this value in (47)

(51) $A = \frac{E_1T_2 - E_2T_1}{T_2 - T_1}$ also, from (49) it is evident that

(52) $E_1/T_1 = E_2/T_2 + 4.55 \log k_2/k_1$

This gives two methods for calculation of E. A is calculated at high temperatures where it is essentially equal to E. If this value of A is taken as the initial E, estimates of E for any other temperature are calculated from equation (51). Or, if k_1 and A are determined at some high temperature, T_1, the value of A can be substituted for E_1 in equation (52) and E calculated for any other temperature T for which k is known. When calculations were made for a number of enzyme reactions, (lipase, emulsin, amylase, pepsin, invertase), it was shown that, although A may vary two to three fold over a wide temperature interval, E remains essentially constant.

The activation energy of an enzyme reaction should be less than is the activation energy of the uncatalyzed reaction - a fact inherent in the concept of a catalyst that is not made use of in the intermediate complex theory. According to Medwedew's theory the substrate molecule receives $h\nu$ ergs of energy from the activated enzyme. Hence, the activation energy of the enzyme-catalyzed reaction, $Ea\phi$, will be less than that of the uncatalyzed, Ea, by $Nh\nu$ ergs per mole, or $Ea = Ea\phi + Nh$, and the temperature coefficient of the enzyme reaction will be $d \ln k / dT = Ea\phi/RT^2 = \frac{Ea - Nh}{RT^2}$

This relationship states that the temperature coefficient of an enzyme catalyzed reaction should be less than the non-enzymic process, an experimental fact known for many years.

The energy of activation of 33 enzyme-catalyzed processes averaged 11,400 cal., whereas the energy of activation of 47 non-catalyzed processes averaged 31,000 cal. The relative number of molecules which would be activated to the point of reaction is given by $e^{-\frac{(11,400 - 31,000)}{RT}}$ or about 10^{14}. This explains the enormous speeding up of reactions by the presence of enzymes. The average activation energy for 12 reactions in the presence of inorganic catalysts is 16,500 cal. By a similar calculation, it can be shown that the enzyme-catalyzed reaction would be expected to be about 10^4 more rapid than the reaction catalyzed by chemical substances; this may be the reason that the chemically catalyzed reaction requires much higher temperatures for a comparable velocity.

According to these data, the cell must have available about 19,600 cal. to produce sufficient activated enzyme molecules to cause the reaction of one mole of substrate. The required energy is smaller than that available for many enzyme-catalyzed processes, notably those involving fermentation or oxidation reactions.

The point which has been previously mentioned that agreement of a particular theory with a few sets of experiment results does not constitute adequate proof of the validity of the theory, is once more illustrated by a comparison of the calculations made by Medwedew and Moelwyn-Hughes on the absolute rate of reaction for the inversion of sucrose by saccharase. Both obtained results equally in agreement with the observed velocity although Medwedew based his calculations on the statistical mechanical hypothesis and Moelwyn-Hughes, on the intermediate complex theory. Clearly, this triumph does not provide a critical test for either theory.

ACTIVATED COMPLEX THEORY

The activated complex theory is an application of the physical-chemical theory of Eyring and others (Eyring, 1935; Glasstone, Laidler and Eyring, 1941) that provides a mechanism for chemical reactions allowing the calculation of their reaction rates from thermodynamical and other physical constants. It has been successfully applied to only a few relatively simple chemical reactions (Daniels, 1943; Eyring, 1944); in general, the calculations and necessary data required would seemingly preclude its application to reactions as complex as those catalyzed by enzymes. Its major use for enzyme chemistry has been to apply it in reverse; that is, knowing the rate of reaction, one can estimate important thermodynamical constants. Knowledge of such constants allows certain inferences to be drawn about the probable mechanism (Stearn, 1938); also the consistency of the results when applied to diverse systems and the agreement of the deductions with others based on a different approach provide support for applicability of the theory and thus indirectly for its validity.

Derivation of the fundamental equation

The experimental evidence that led to the development of a suitable mechanism for application of the theory to biological systems was derived primarily from studies on luminescence in bacteria and other organisms (Eyring and Magee, 1942; Brown, Johnson and Marsland, 1942), but a more general statement of the theory has been recently furnished by Johnson and Lewin, (1946, p. 62). The theory for calculation of the absolute rate of a reaction proposes that a chemical reaction takes place after the formation from the reactants of an activated complex that decomposes into the products. The rate of reaction is given by the expression:

(53) $k' = \underline{k}\,(kT/h)K^{\ddagger}$ in which

k' - specific rate of reaction

$\underline{k}$ - "transmission coefficient," a constant that measures the probability that the activated complex having broken apart will not reconstitute itself; usually taken as equal to unity.

k - Boltzmann's constant, R/N, 1.37×10^{-16} erg/degree

h - Planck's constant, 6.55×10^{-27} erg-second

T - absolute temperature

$K^{\ddagger}$ - equilibrium constant between reactants and activated complex.

From thermodynamics:

(54) $- RT \ln K^{\ddagger} = \Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} = \Delta E^{\ddagger} + P\Delta V^{\ddagger} - T\Delta S^{\ddagger}$ in which the daggers indicate that the thermodynamic constants, E, the energy, F, the free energy, S, the entropy, H, the heat, V, the volume, and P, the pressure, refer to the activation reaction. For example, $\Delta F^{\ddagger}$ is the difference between the free energy of the activated complex and the reactants. For convenience, in the following we shall write $\underline{k}(k/h)$ as C and use the convention that $\underline{\exp x}$ means e^x. Then from the thermodynamical relationships, equation (53) can be written:

$$(55)\quad k' = CT \exp -\Delta F^{\ddagger}/RT = CT \exp -\Delta H^{\ddagger}/RT \exp \Delta S^{\ddagger}/R$$

$$= CT \exp -\Delta E^{\ddagger}/RT \exp - P\Delta V^{\ddagger}/RT \exp \Delta S^{\ddagger}/R$$

Consider an enzyme reaction with specific rate of reaction, k_1, under control of an enzyme (En) acting on substrate (X):

$$(56)\quad k_1 = \underline{k}\,(kT/h)\,K^{\ddagger}(En)\,(X) = CT\,(En)\,(X) \exp \Delta H^{\ddagger}/RT \exp \Delta S^{\ddagger}/R$$

$$= CT\,(En)\,(X) \exp -\Delta E^{\ddagger}/RT \exp - P\Delta V^{\ddagger}/RT \exp \Delta S^{\ddagger}/R$$

The native active enzyme is considered to be in reversible thermal equilibrium with a denatured inactive form:

(57) $En \longleftrightarrow Ed$ with equilibrium constant $K_1 = \exp -\Delta H_1/RT \exp \Delta S_1/R$. If Eo is total enzyme equal to En + Ed, then it is readily shown that

$$(58)\quad En = Eo/(1+K_1) = \frac{Eo}{1 + \exp -\Delta H_1/RT \exp \Delta S_1/R}$$

$$= \frac{Eo}{1 + \exp - \Delta E_1/RT \exp - P\Delta V_1/RT \exp \Delta S_1/R}$$

in which the subscript 1 refers to the thermodynamic constants of the equilibrium (57). If the rate of reaction is represented by I, then

$$(59)\quad I_1 = sk'(En)\ (X) = \frac{sk'\ (E_o)\ \ (X)}{1 + K_1}$$

$$= \frac{sk\ (kT/h)\ (X)\ (Eo)\ \exp - \Delta H\ddagger/RT \exp \Delta S\ddagger/R}{1 + \exp - \Delta H_1/RT \exp \Delta S_1/R}$$

In (59) s is merely a proportionality constant to take care of the units used in the measurements; k/h is a constant; Eo and (X) are assumed to be essentially constant as is $\Delta S\ddagger/R$ so that equation (59) can be written with all these as one constant, c',

$$(60)\quad I_1 = \frac{c'T \exp - \Delta H\ddagger/RT}{1 + \exp - \Delta H_1/RT \exp \Delta S_1/R}$$

$$(61)\quad I_1 = \frac{c'T \exp - \Delta E\ddagger/RT \exp - P\Delta V\ddagger/RT}{1 + \exp - \Delta E_1/RT \exp - P\Delta V_1/RT \exp \Delta S_1/R}$$

Effect of temperature

Equations (60) and (61) are the fundamental ones that describe how the rate of an enzyme reaction changes with temperature and pressure. Before illustrating their use, the method of calculating the constants must be considered. As the pressure is usually constant in most enzymatic reactions, equation (60) is the preferred form. The constants in this equation are estimated from the curve formed when the log rate of reaction is plotted against the reciprocal of the absolute temperature. As is illustrated in Figure 6, it is usually convenient to plot the log of the relative rate of reaction (optimum equal to 100) against 1/T. $\Delta H\ddagger$, taken equal to the μ value of the Arrhenius equation discussed on page 18, is obtained by multiplying by 4.6 the slope of the line that best fits the experiment points from the low points to those where it deviates in approaching the optimum. ΔH_1 is more difficult to establish but can be estimated by adding, disregarding the signs, the value of $\Delta H\ddagger$ to that obtained when the decreasing slope in the region between 20-40% of the maximum rate is multiplied by 4.6 calories. A more nearly correct figure for ΔH_1 may be arrived at by calculating the entire curve using figures slightly higher and lower than the one first estimated to see which best fits all the experimental points. The theory presumes that the enzyme-catalyzed reaction obeys the Arrhenius equation over the entire temperature range (see Figure 6) and that denaturation interferes to an extent sufficient to yield a positive slope at higher temperatures. When $\Delta H\ddagger$ and ΔH_1 are determined, ΔS_1 is calculated from

$$(62)\quad K_1 = \exp - \Delta H_1/RT \exp \Delta S_1/R$$

taking advantage of the fact that at the optimum temperature (I = 100)

$$(63)\quad K_{1max} = \frac{\Delta H\ddagger + 600}{\Delta H_1 - \Delta H\ddagger - 600} -$$ (Koffler, Johnson, and Wilson, 1947)

Once ΔS_1 is known, K_1 can be calculated for all other temperatures; then by substituting the estimated values of $\Delta H^{\ddagger}$, ΔH_1 and ΔS_1 in equation (60) c' is determined.

Example 1.

From the slopes of the 0.0 line in Figure 6, $\Delta H^{\ddagger}$ is estimated to be 13,400 calories and ΔH_1, 96,000 calories. The optimum temperature is 37° C. (I = 100 at T = 310). Accordingly,

$$K_{1max} = \frac{13,400 + 600}{96,000 - 13,400 - 600} = \frac{14,000}{82,000} = 0.1707 = \exp -1.767$$

Therefore from (62) exp - 1.767 = exp (48,000/310)(exp $\Delta S_1/2$).

exp $\Delta S_1/2$ = exp (153.07) or ΔS_1 = 306.14; substituting these values in equation (60),

$$100 = \frac{c' \ 310 \exp - (13,400/620}{1 + \exp (-48,000/310) \exp (153.07)} = \frac{c' \ 310 \exp -21.61}{1 + \exp (-154.84) \exp (153.07)}$$

$$= \frac{310 \ c' \exp -21.61}{1 + \exp -1.77} = \frac{310 \ c' \exp -21.61}{1 + 0.1703}$$

$$c' = \frac{117.03 \exp 21.61}{310} = 0.3775 \exp 21.61, \quad \text{and therefore}$$

$$(60') \quad I_1 = \frac{0.3775 \ T \exp 21.61 \exp (-6,700/T)}{1 + \exp (153.07) \exp (-48,000/T)}$$ from which the solid 0.0 line in Figure 6 is constructed.

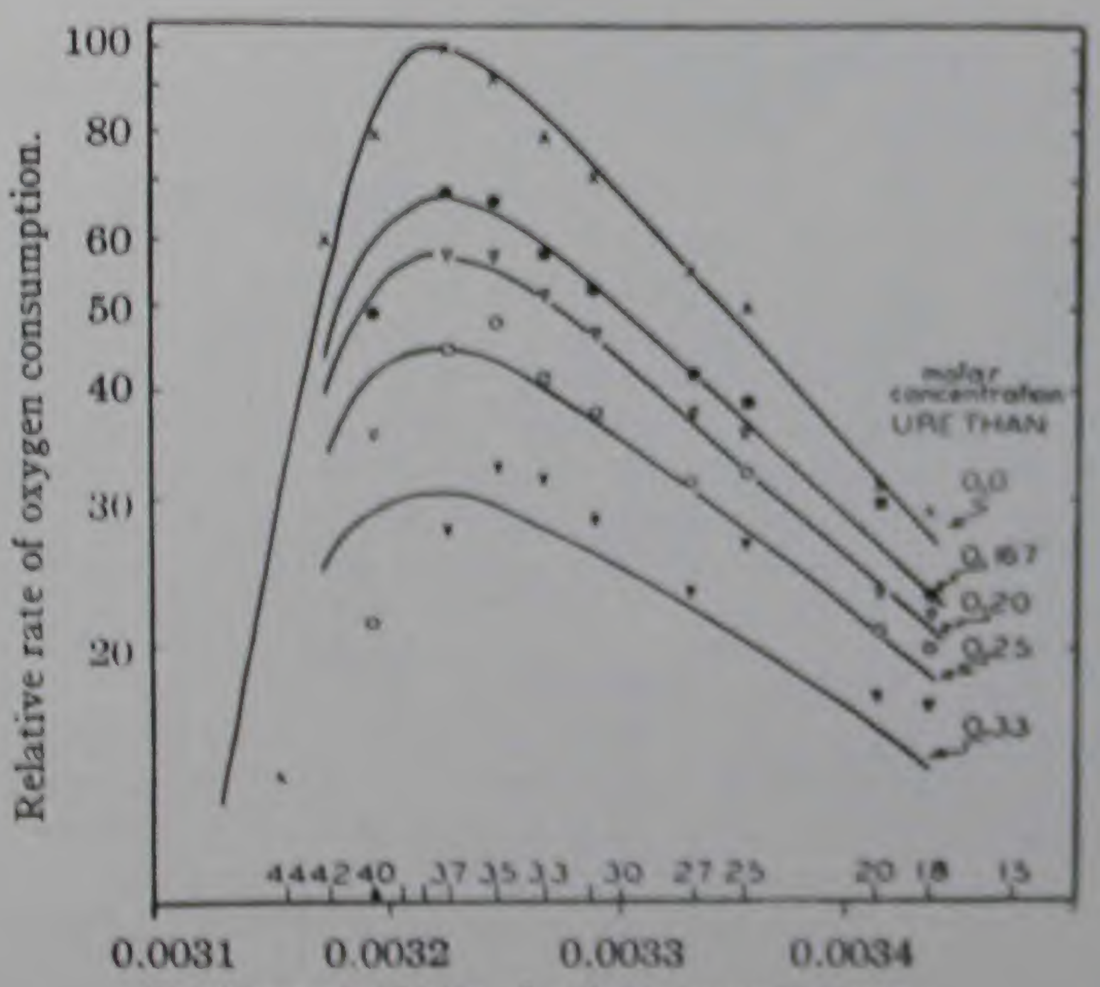


Figure 6

Relation between temperature and rate of oxygen uptake by Rhizobium trifolii (Koffler, Johnson and Wilson, 1947).

The solid lines are drawn from equations (60') and (69') as described in the text.

Typical values of $\Delta H^{\ddagger}$, ΔH_1 and ΔS_1 for various enzymatic processes in bacteria are summarized in Table 1. The cited references should be consulted for interpretation of these in terms of the specific processes under investigation. In general, they agree with similar data for proteins other than enzymes (Eyring and Stern, 1939) and thus are consistent with the views of activation and denaturation required by the proposed mechanism.

TABLE I

Typical values for heats of activation and for heats and entropy of denaturation

Process	$\Delta H^{\ddagger}$ kg-cal	ΔH_1 kg-cal	ΔS_1 e.u.	Reference
Luminescence				
Vibrio phosphorescens	28.4	91.2	300	Johnson, Eyring and Williams, 1942
Photobacterium phosphoreum	15.6	71.0	239	
Achromobacter Harveyi	27.0	69.6	228	
A. Fischeri	34.8	83.8	279	
Growth - E. coli	15.6	150.0	476	Johnson and Lewin, 1946
Dehydrogenation - Rhizobium trifolii	13.6	126.0	400	Koffler, Johnson and Wilson, 1947
Respiration - R. trifolii	13.4	96.0	306	

Action of an inhibitor

The usefulness of the theory of absolute reaction rate as summarized in equations (60) and (61) has been greatly increased by extension to include the activity of inhibitors. The necessary equations for the extension can be derived as follows (Johnson, Eyring and Williams, 1942):

Let the following scheme represent the combination of r molecules of inhibitor Y with the native enzyme, En, and/or s molecules with the denatured enzyme, Ed:

$$\begin{array}{ccc} rY + En & \xleftrightarrow{K_1} & Ed + rY \\ K_2 \big\downarrow\big\uparrow & & \big\uparrow\big\downarrow K_3 \\ En\text{-}Yr & & Ed\text{-}Ys + (r - s)\,Y \end{array}$$

$Eo = En + Ed + En\text{-}Yr + Ed\text{-}Ys$ and the equilibrium constants of the various reactions are:

$$K_1 = (Ed)/(En) \qquad K_2 = (En\text{-}Yr)/(En)(Y^r)$$

$$K_3 = (Ed\text{-}Ys/(Ed)(Y^s) \qquad \text{and } K_1 K_3 = (Ed\text{-}Ys)/(En)(Y^s)$$

From these equations $Eo = (En) + (En)K_1 + (En)(Y^r)\,K_2 + (En)\,K_1K_3\,(Y^s)$
and $En = Eo/(1 + K_1 + K_2\,Y^r + K_1K_3\,Y^s)$

If the velocity in the presence of inhibitor is I_2, then

$$(64) \quad I_2 = \frac{sk'\,(Eo)\,(X)}{1 + K_1 + K_2\,Y^r + K_1K_3\,Y^s}$$

From this general equation of inhibition, three types have been differentiated:

Type I: The inhibitor combines indiscriminately with both native and denatured enzyme: $K_2 = K_3$ and $r = s$

Equation (64) then reduces to

$$(65) \quad I_2 = sk'(Eo)(X) \,/\, (1 + K_1)(1 + K_2Y^r)$$

Dividing equation (59) by (65) leads to

$$(66)\quad I_1/I_2 = 1 + K_2Y^r = 1 + (\exp - \Delta H_2/RT)(\exp \Delta S_2/R)(Y^r)$$
$$= 1 + (\exp - \Delta E_2/RT)(\exp - P\Delta V_2/RT)(\exp \Delta S_2/R)(Y^r)$$

$$(67)\quad \log (I_1/I_2 - 1) = -\Delta H_2/2.3\ RT + \Delta S_2/2.3R + r \log Y$$
$$= -\Delta E_2/2.3\ RT - P\Delta V_2/2.3\ RT + \Delta S_2/2.3\ R + r \log Y$$

Equation (67) shows that if $\log (I_1/I_2 - 1)$ is plotted successively against $1/T$, $\log Y$ and P, the slopes of the resulting lines will determine ΔH_2, $\underline{r}$ and ΔV_2.

Type II: The inhibitor combines only with the denatured form of the enzyme: $\underline{K_2} = 0$.

Since now $En = Eo/(1 + K_1 + K_1K_3\ Y^s)$

$$(68)\quad I_2 = sk'(Eo)(X) \,/\, (1 + K_1 + K_1K_3\ Y^s)$$

$$(69)\quad I_1/I_2 = (1 + K_1 + K_1K_3\ Y^s)/(1 + K_1) = 1 + K_1K_3\ Y^s/(1 + K_1)$$

$$(70)\quad I_1/I_2 - 1 = \frac{K_1K_3}{1 + K_1} Y^s = K_3\ Y^s/(1 + 1/K_1)$$

$$(71)\quad (I_1/I_2 - 1)(1 + 1/K_1) = K_3Y^s = (\exp - \Delta H_3/RT)(\exp \Delta S_3/R)(Y^s)$$

$$(72)\quad \log (I_1/I_2 - 1)(1 + 1/K_1) = -\Delta H_3/2.3\ RT + \Delta S_3/2.3\ R + s \log Y$$

$$(73)\quad = -\Delta E_3/2.3\ RT - P\Delta V_3/2.3\ RT + \Delta S_3/2.3\ R + s \log Y$$

Analogous to type I, if $\log (I_1/I_2 - 1)(1 + 1/K_1)$ is plotted against $1/T$, $\log Y$ and P, the slopes of the resulting straight lines estimate ΔH_3, $\underline{s}$ and ΔV_3. From (70) it is evident also that just as in Type I, if $\log (I_1/I_2 - 1)$ is plotted against $\log Y$ the slope is $\underline{s}$, the number of molecules combining with inhibitor. The intercept, however, is not simply the log of the dissociation constant as in Type I but is $\log K_1K_3/(1 + K_1)$.

Type III: The inhibitor combines only with native enzyme: $\underline{K_3} = 0$.

Although the final equation for this type of inhibition is slightly different, it cannot be distinguished from Type II on the basis of kinetic data (McElroy, 1943; 1947).

The inhibition of respiration in Rhizobium trifolii by urethan is evidently Type II as is indicated by the linearity of the points when $\log (I_1/I_2 - 1)(1 + 1/K_1)$ is plotted against $1/T$ (Figure 7). From the slopes of the lines in the figure ΔH_3 is estimated as -85,000 cal. The number of molecule $\underline{s}$, combining with the enzyme increases from 1.3 to 3.9 as the temperature increases from 18° to 40° C. (Figure 8). This is interpreted that, as the enzyme is denatured, bonds open with which urethan combines. Assuming an average value of 2.25 will not cause serious error in the estimate of ΔS_3 but will markedly influence the course of the curves calculated for various concentrations of urethan (solid lines in Figure 6).

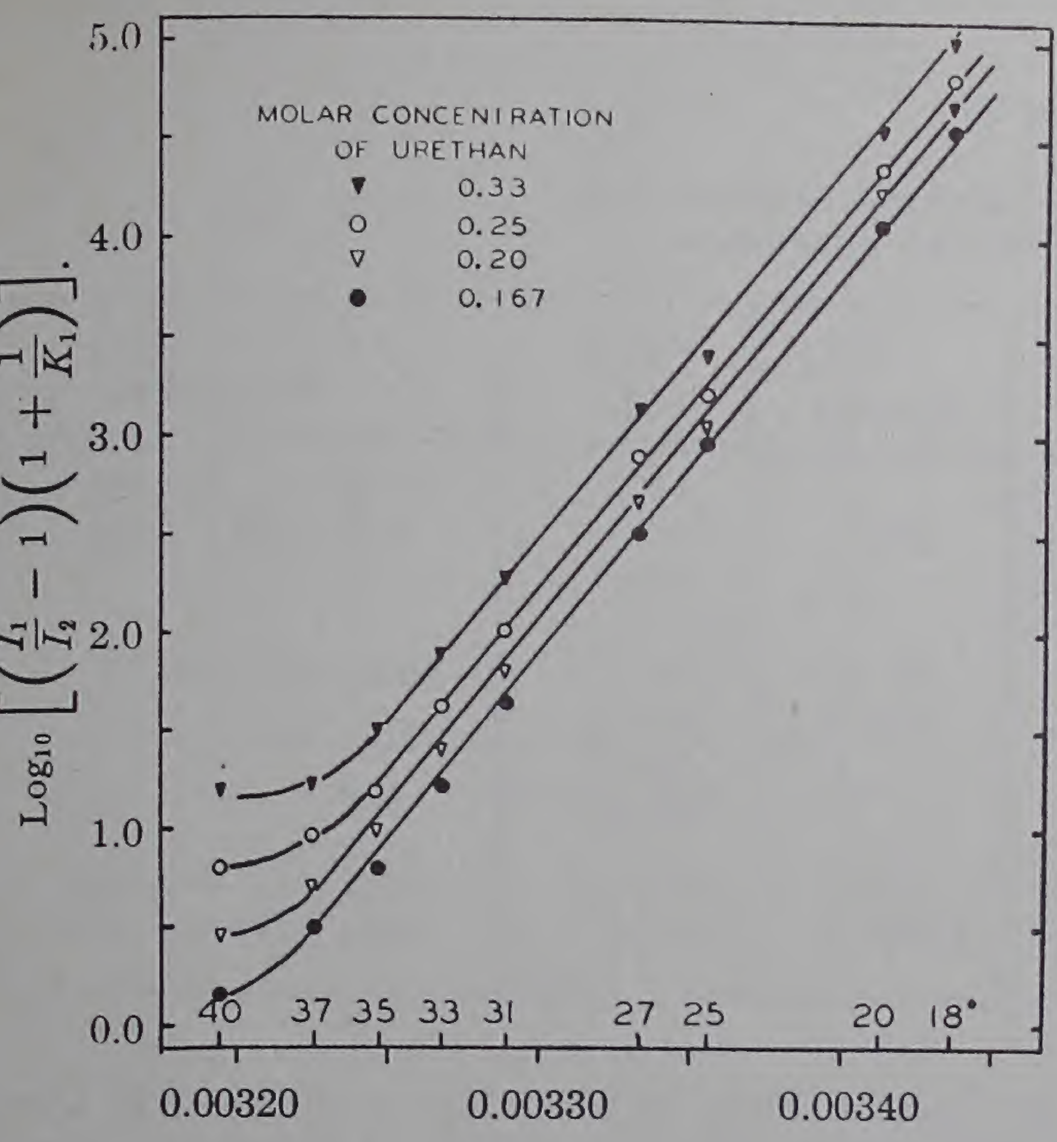


Figure 7

Inhibition by urethan of oxygen uptake by R. trifolii plotted according to equation (72) with 1/T as the independent variable

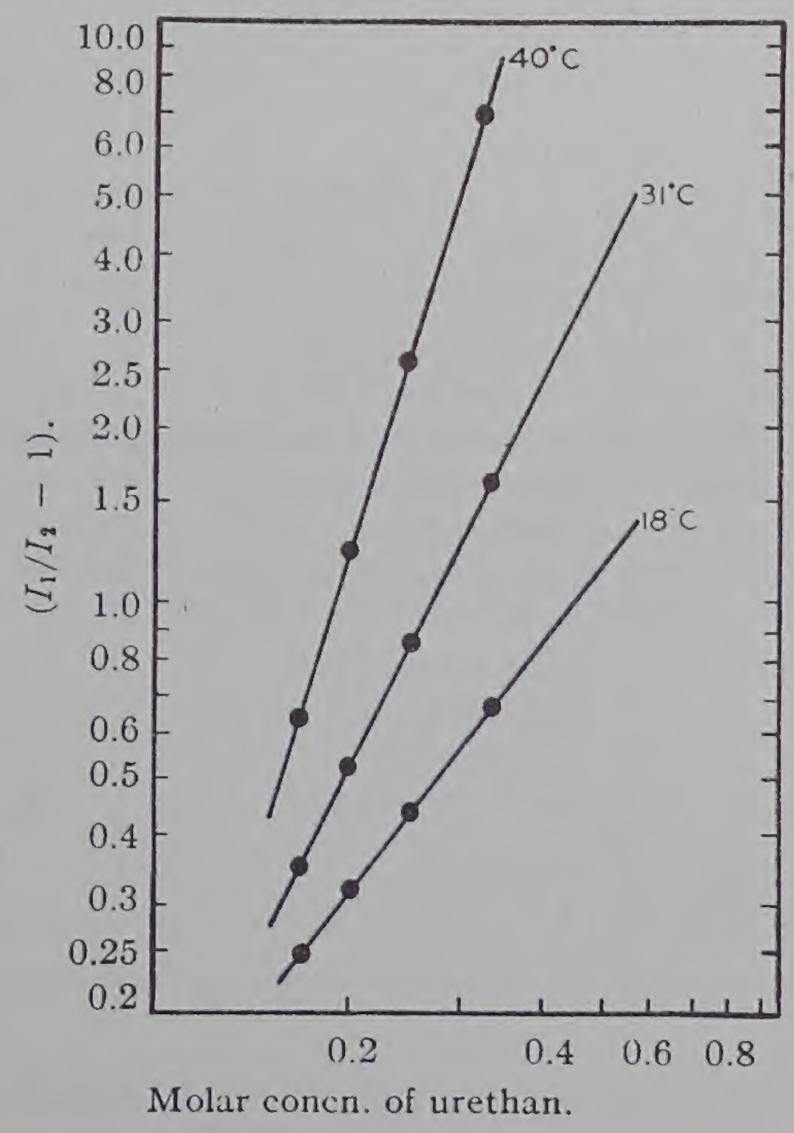


Figure 8

Inhibition of oxygen uptake of R. trifolii as function of urethan concentration. Plotted according to equation (70).

Example 2.

At 27° C. (T = 300) and at a urethan concentration of 0.2 M it was experimentally determined that $(I_1/I_2 - 1) = 0.455$. From Example 1 it was known that $K_1 = \exp(-96{,}000/600)\exp(306.14/2) = \exp -6.93$. Since $(0.2)^{2.25} = \exp - 3.62$, then $(1 + 1/K_1)(I_1/I_2 - 1) = (1 + \exp 6.93)(0.455) = (1 + 1023)(0.455)$

$$= 466 = \exp 6.15$$

From equation (71) $\exp 6.15 = \exp (85{,}000/600) \exp (\Delta S_3/2) \exp (- 3.62)$

$$\exp \Delta S_3/2 = \exp - 131.9 \qquad \Delta S_3 = - 263.8 \text{ e. u.}$$

Substituting in (69)

$$(69') \quad I_2 = \frac{I_1}{1 + \dfrac{\exp(-48{,}000/T)\exp(153.07)\exp(42{,}500/T)\exp(-131.9)}{1+\exp(-48{,}000/T)\exp(153.07)}\,U^{2.25}}$$

$$= \frac{I_1}{1 + \dfrac{\exp(-5{,}500\,T)\exp 21.17}{1+\exp(-48{,}000/T)\exp 153.07}\,U^{2.25}}$$

From equation (69') the solid lines in Figure 6 corresponding to different concentrations of urethan (U) can be calculated.

Typical values for these thermodynamic constants of inhibition are summarized in Table 2.

TABLE II

Heats of reaction and entropy changes for inhibition of bacterial processes

Process	Inhibitor	$-\Delta H_2$ kg-cal.	$-\Delta H_3$ kg-cal.	$-\Delta S$ e.u.	Reference
Luminescence	Sulfanilamide	17.3		39.2	Johnson, Eyring and Williams, 1942
	Urethan		70.0	204	
	para-aminobenzoic acid	18.2		50	
	Phenobarbital		44.2	146.5	McElroy (1943)
	Chloretone		49.2	166.5	
	Benzamide	12.0		40.1	
	Salicylamide	18.9		65.8	
Respiration MB reduction	Urethan		85.0 118.0	263.8 366.2	Koffler, Johnson and Wilson, 1947

Apparently, inhibitors generally classified as "competitive" are of Type I with $-\Delta H_2$ about 15-20 kg-cal. and $-\Delta S_2$ around 50 e. u., and frequently, but not always, r equal to 1 or 2. "Non-competitive" inhibitors appear to be Type II with a considerable higher heat of reaction and increase in entropy; s may vary continuously with temperature. Additional investigations of various processes and inhibitors are needed to verify these generalizations.

Pressure

The validity of the application of the physical-chemical theory based primarily on thermodynamical considerations receives considerable support from the results dealing with the effect of pressure on the rate of reaction. The other mechanisms neglect the influence of pressure, and experimentally it has been disregarded by making the studies at a constant pressure of one atmosphere. Equations (61, 67, and 73) predict that a change in pressure will alter the effect of both temperature and inhibitors. By ingenious techniques that allowed the study of reactions under pressures up to 7,000 lbs./sq. in. the Princeton group of workers has verified this prediction (Brown, Johnson, Marsland, 1942; Johnson, Brown and Marsland, 1942a; Johnson, Eyring and Williams, 1942; Johnson and Lewin, 1946). The interpretation of the influence of pressure in terms of the proposed mechanism is that both activation and denaturation of the native enzyme molecule lead to an increase in volume. At low temperatures where the En + X $\longleftrightarrow$ En-X equilibrium dominates, pressure will decrease the rate of reaction by restraining the formation of the activated complex. At higher temperatures this restraint still obtains but is overshadowed by the influence of the En $\longleftrightarrow$ Ed equilibrium. Here, pressure furthers the formation of En, thus providing more enzyme for activation with a consequent increase in the rate of reaction (see Figure 9).

As is illustrated in Figure 10, application of pressure will likewise overcome in part the effect of certain inhibitors, and a similar explanation is offered. Support for the interpretation and thus indirectly for the entire theory and its attendant mechanisms is given by the study of the effect of pressure and temperature on enzymic and other proteins (Eyring and Stearn, 1939; Stearn and Eyring, 1941; Johnson and Campbell, 1946; Johnson and Wright, 1946; Eyring, Johnson and Gensler, 1946).

The value of $\Delta V^{\ddagger}$ can be estimated from the slope of line obtained by plotting log I_1 against P (see equation 61); ΔV_1 is somewhat more difficult to estimate, but by application of certain corrections this can be obtained as well. Brown, Johnson and Marsland (1942) report $\Delta V^{\ddagger}$ for luminescence to be about 58 cc per mole at 0° C. and V_1 = 116 cc per mole at 35° C. These figures are in reasonable agreement with those of Eyring and

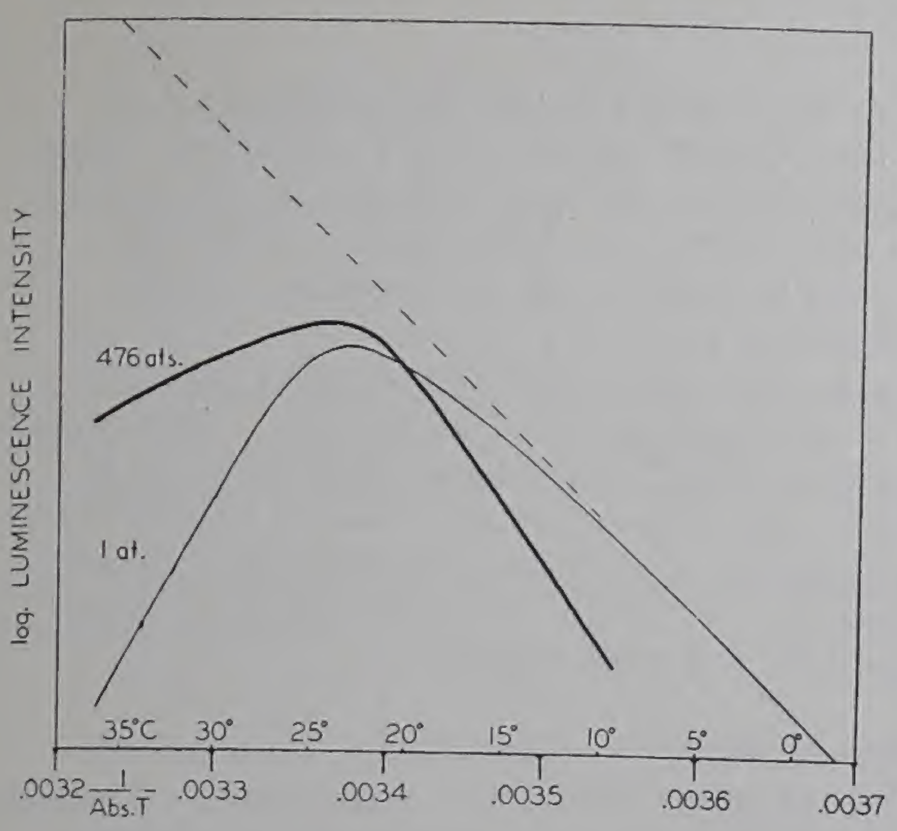


Figure 9

Relation between temperature and luminescence at 1 and 476 atm. pressure. The extrapolated broken line represents luminescence when corrected for reversible denaturation (Johnson, Brown and Marsland, 1942).

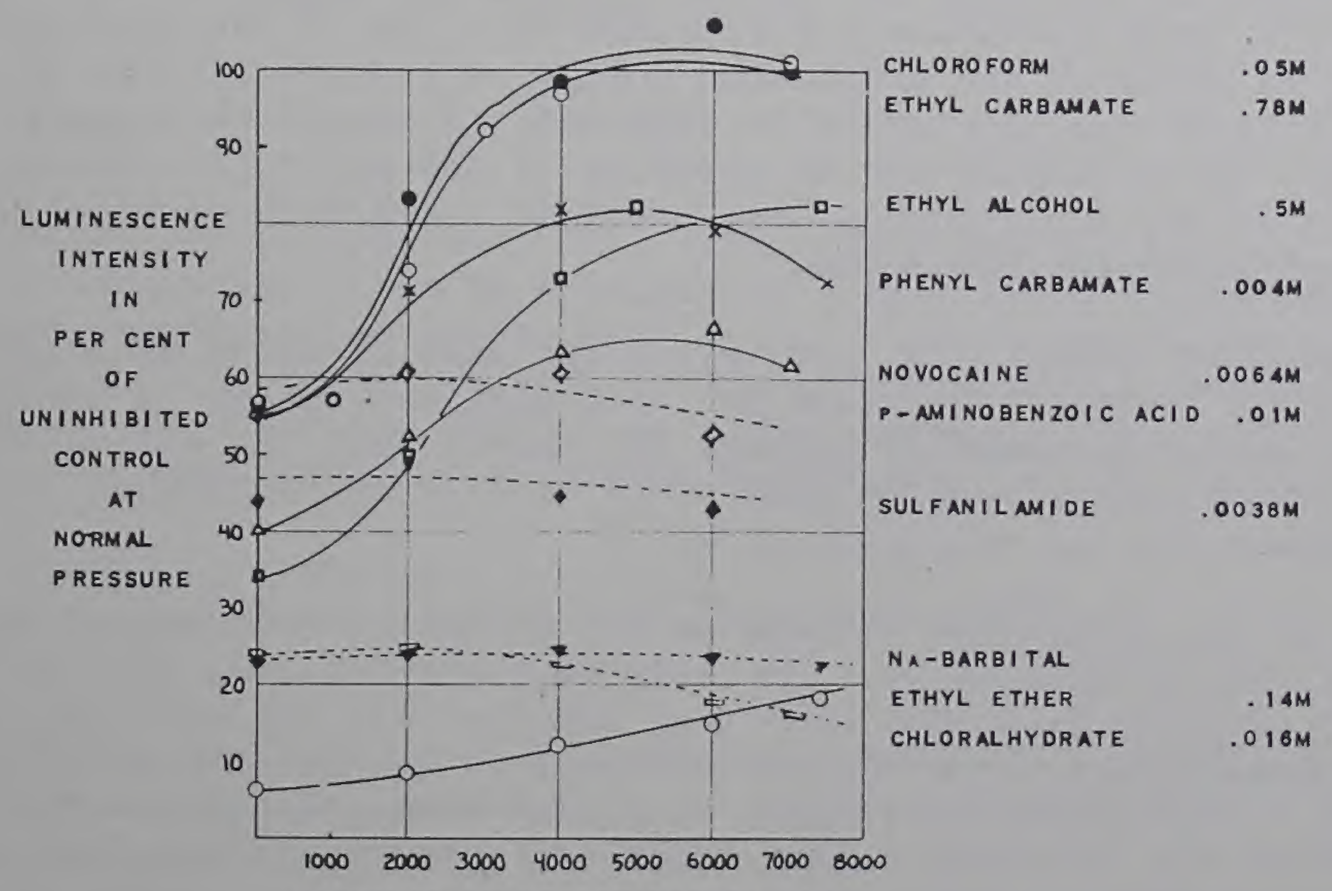


Figure 10

Effect of pressure at 17-18° C. on luminescence by P. phosphoreum in presence of various inhibitors. Inhibitions not sensitive to pressures are in dotted lines (Johnson, Brown and Marsland, 1942a).

Magee (1942) who used a somewhat different method for their estimation, one that provided a correction for changes in ΔV with temperature. In the presence of inhibitors the proper method of plotting is log $(I_1/I_2 - 1)$ against P; Johnson and Schneyer (1944) found as a first approximation 20 cc per mole for the combination with quinine of the enzyme controlling luminescence in P. phosphoreum. The interpretation of these findings is that not only activation and denaturation but also combination with inhibitor increase the volume of the protein molecule of the enzyme.

General implications of the theory

The many noteworthy and interesting inferences made regarding specific details of various enzymatic reactions based on application of the theory of absolute reaction rate can be appreciated only through study of the individual applications listed in the references. More general discussions of the implications are given in the papers of Stearn (1938), Sizer (1943), Johnson, Eyring, Steblay and others (1945), Rysselberghe (1944), Johnson (1947) and McElroy (1947). Stearn (1938) suggests possible mechanisms based on the approach of a dipole (or a charge on a large enzyme) to the reacting molecules with a resulting change in configuration and consequently in the energy of activation. The quantitative aspects of the change will depend on the various configurations that may be assumed. Through construction of specific energy curves, he demonstrates that a significant lowering of the energy of activation can occur and believes that such mechanisms are more probable than those previous suggested based on loosening of bonds by distortion. Three other conclusions of Stearn's are of general significance for the theory:

(1) Activation in the absence of enzyme is accompanied by a very small loss in entropy, but this loss is quite appreciable for enzyme-catalyzed reactions. Stearn suggests that this would be expected if an enzyme-substrate compound is formed. By uniting with the substrate the enzyme decreases the entropy of the system, and this loss tends to defeat its catalytic function. That is, the enzyme is "self-interfering" in that it loses "randomness". Any catalytic activity must be in excess of that necessary to offset the loss in entropy of the system. In the acid inversion of sucrose, for example, ΔH is about 26,000 cal. and $\underline{k}$ is approximately 10^{-5}. With invertase ΔH is 8,600 cal., so if the only effect of the enzyme is to decrease the activation energy, a $\underline{k}$ value of over 10 million for the enzyme-catalyzed reaction would be expected. Actually it is only 10^{-5} to 10^{-6}. Apparently, the catalysis due to lowering of the activation energy is overcome by a decrease in ΔS of 50-60 cal./deg./mole in comparison with no loss of entropy in the acid-catalyzed inversion.

(2) Unlike Medwedew, Stearn does not reject the idea of union of enzyme and substrate. Stearn (1938) states, "This does not mean that such union is unnecessary for catalytic action by means of enzymes but merely that the effect of the enzyme on the activation energy must be sufficient to overcome this loss in entropy with some to spare for net catalytic effect."

(3) In spite of the criticisms leveled at the Michaelis constant calculated in the usual way, it may possess real thermodynamic significance for the reaction. Values of unity to over 1000 have been reported for the affinity constants of enzyme and substrate (the affinity constant is the reciprocal of the Michaelis constant). Such values correspond to a free energy decrease of 0 to -4000 cal. The question is: are such values consistent with other knowledge concerned with the thermodynamics of enzyme reactions? From the calculations of ΔS for a large number of enzyme reactions, it appeared that the loss in entropy would be 30 units or more: ΔH for these reactions ranged from -4000 to -13,450 cal. Accordingly, at 27° C., $\Delta F = \Delta H - T\Delta S = \begin{cases} -4000 \\ \text{to} \\ -13{,}450 \end{cases} + (300)(30) = -4500 \text{ to zero}$ up
cal. This rough approximation indicates that the observed values of the Michaelis constant are fairly consistent with the "quasi-thermodynamics" of enzyme reactions.

CALCULATION OF THE ABSOLUTE RATE OF REACTION

As indicated in the preceding section, a major objective of chemical kinetics is to provide suitable methods for the calculation of the absolute rate of a reaction from the physical-chemical properties of the system. This section will discuss some of the efforts to achieve this goal based on two of the proposed enzyme mechanisms.

COLLISION THEORY

Moelwyn-Hughes (1933) employed the data of Nelson and Vosburg (1917) on the hydrolysis of sucrose by a crude preparation of saccharase for a typical calculation based on the unmodified collision theory (Hinshelwood's corrections neglected). The details are:

Concentration of sucrose - 5.0/100 ml.

Concentration of crude saccharase - 0.06 g/100 ml. From its activity it was estimated that this preparation contained 2.5×10^{-5} grams of pure saccharase per 100 ml. Diffusion experiments indicated that saccharase had a molecular weight of about 50,000.

The number of collisions between substrate and enzyme is estimated from the gas collision formula:

$$(74) \quad Z = n_1 n_2 \frac{\sigma_1 + \sigma_2}{2} \left\{ 8\pi RT \left(\frac{1}{M_1} + \frac{1}{M_2} \right) \right\}^{\frac{1}{2}}$$

n_1 - number of molecules of sucrose per ml., 8.86×10^{19}
n_2 - number of molecules of saccharase per ml., 3.03×10^{12}
σ_1 - diameter of sucrose molecule 9.0×10^{-8} cm.
σ_2 - diameter of saccharase molecule 5.5×10^{-7} cm.
R - 8.31×10^{7} ergs. per degree
T - 310° K.
M_1 - 342
M_2 - 50,000

Substituting these values in equation (74), 1.21×10^{24} per ml. per sec. is obtained for Z. The effective collisions are given by multiplying Z by $e^{-E/RT}$. $\underline{E}$ has been estimated as 8,700 cal.: hence, the fraction of the collisions in which the energy is greater than 8,700 cal. is $e^{-8,700/2 \times 310}$ or 7.0×10^{-7}. This figure multiplied by Z gives 8.47×10^{17} as the total number of activating collisions. The observed value of $\underline{k}$ is 2.1×10^{-4} per sec.; this means that the number of molecules decomposing per ml. per sec. is

$$\frac{0.05 \times (6.06 \times 10^{23})}{342} \times (2.1 \times 10^{-4}) = 1.86 \times 10^{16}.$$

Comparison of the observed with the calculated value indicates that of each 46 "theoretical" activating collisions, on the average only one is effective in promoting decomposition. The apparent reason for this low efficiency is that not all of the surface of the enzyme molecule is catalytically active. If the reasonable assumption is made that the probability of a sucrose molecule hitting an "active" center is equal to $\frac{\sigma_1^2}{\sigma_2^2}$ or 1/37, the agreement is most satisfactory.

Although Moelwyn-Hughes (1937) prefers the chain reaction mechanism for the decomposition of hydrogen peroxide by catalase, he demonstrates in the following calculation that the observed rate of reaction can be satisfactorily accounted for on a purely collisional basis.

If the enzyme is regarded as a "wall", the number of H_2O_2 molecules striking an area, A, is equal to

$$A\, n_s \sqrt{kT/2\pi m_s}$$

where n_s - molecules of substrate of radius, $\underline{r} = 3 \times 10^{-8}$ cm.
n_e - molecules of enzyme
k - Boltzmann gas constant
m_s - weight of one molecule of H_2O_2 $34/(6.06 \times 10^{23})$

The surface of the enzyme per ml. is $\frac{4}{3}\pi R^2 n_e$, and the total number of collisions per sec. will be $n_e n_s \frac{4}{3}\pi R^2 \sqrt{kT/2\pi m_s}$. The probability that a substrate molecule will hit the enzyme on an active spot may be taken as:

$$P = \frac{\pi r^2}{\frac{4}{3}\pi R^2} = \frac{3 r^2}{4 R^2}.$$ Assuming that each activating collision is effective, the rate of reaction will be $- \frac{dn_s}{dt} = n_s n_e r^2 \sqrt{\pi kT/2m_s} \exp -E/RT$. The average of two independent closely agreeing values for E is 4,240 cal.: hence, the velocity constant is,

$$k' = - \frac{dn_s}{dt} \quad \frac{1}{n_s n_e} = r^2 \sqrt{\pi \frac{kT}{2m_s}}\, e^{-4240/546} = 12.7 \times 10^{-15} \text{ cc./molecule/sec.}$$

As the observed value for k' is 9×10^{-15} cc./molecule/sec., the collision hypothesis will explain the rate of decomposition.

ACTIVATED COMPLEX THEORY

Since this statistical mechanical theory gives the proper equilibrium constant ($K^{\ddagger}$), it should yield equally good values for reaction velocities for both the forward and reverse reactions. Data for test of the ability of the theory to predict both reaction velocities are available for the heat inactivation of trypsin. The calculations of Stearn (1938) are:

(a) Considering first the forward reaction,

Trypsin ⟶ Inactivated trypsin $k' = 2.83 \times 10^{-5}$ at 50° C.
$E = 40{,}800$ cal. (Pace, 1930)

The "E value" is the Arrhenius activation energy determined in the usual way.

$\Delta H^{\ddagger}$ is calculated from $\Delta H^{\ddagger} = E^{\ddagger} - RT = 40{,}800 - (2)(323) = 40{,}160$ cal.

$\Delta S^{\ddagger}$ is determined from equation (55):

$$2.83 \times 10^{-5} = e^{\Delta S^{\ddagger}/R}\, e^{-\Delta H^{\ddagger}/RT} \qquad \left[\frac{(1.37 \times 10^{-16})(323)}{(6.55 \times 10^{27})}\right]$$

$$2.3 \log (2.83 \times 10^{-5}) = \Delta S^{\ddagger}/R - 62.35 + 2.3 \log (6.76 \times 10^{12})$$

$$-\Delta S^{\ddagger} = 2\,(10.45 - 62.35 + 29.55) = 44.7 \text{ cal./deg./mole}$$

(b) Now considering the equilibrium reaction,

Active trypsin ⟷ Inactive trypsin $K \left(\frac{\text{Inactive}}{\text{Active}}\right) = 7.2$ at 50° C.

$\Delta H = 67{,}600$ cal. (Anson and Mirsky, 1934)

Since $-\Delta F = RT \ln K$

$= (646) \ln 7.2 = 1269$ cal./mole

And $\Delta F = \Delta H - T\Delta S$

$$\Delta S = \frac{67{,}600 + 1269}{323} = 213.1 \text{ cal./degree/mole.}$$

Taken together, the data from reactions (a) and (b) appear to be irreconcilable since the reverse reaction, Inactive trypsin ⟶ active trypsin, has a heat of reaction equal to, 40,160 - 67,600 = -27,440 cal. A negative heat of reaction would predict an enormous reactive velocity, whereas the actual velocity is only 1/7.2 times that of the forward reaction which has a positive heat of reaction of about 40,000 cal. The circumstance which acts as a brake on the velocity of the reverse reaction is the extremely high loss in entropy equal to, 44.7 - 213.1 = - 168.4 cal./deg./mole. The velocity of the reverse reaction can be calculated from equation (55):

$$k' = e^{-168.4/2} \quad e^{27440/646} \quad \frac{(1.37 \times 10^{-16})(323)}{6.55 \times 10^{-27}} = 4.3 \times 10^{-6}$$

The "observed" value for k for the reverse reaction calculated from K and the k' of the forward reaction is $(2.83 \times 10^{-5}) / 7.2 = 3.9 \times 10^{-6}$.

MISCELLANEOUS

Wilson and Burris (1947) calculated an absolute rate of reaction based on collision theory for the fixation of molecular nitrogen by Azotobacter vinelandii. Studies of inhibition by physostigmine of the hydrolysis of choline esters by the esterase, enabled Esson and Stedman (1936) and Straus and Goldstein (1943) to estimate "turnover numbers," which are essentially estimates of the absolute rates of reaction. Other turnover numbers for various enzymatic reactions are discussed by Green (1940) and Sumner and Somers (1943).

ADDENDUM: THE EFFECT OF pH ON ENZYMES

by Marvin J. Johnson

Enzyme action may be affected by pH in two ways, since both the stability and the activity of the enzyme are functions of pH. The effect of pH on enzyme stability will be considered first. Most, if not all, enzymes undergo irreversible denaturation in very acid and very alkaline solutions. The pH values at which this occurs vary with the enzyme. Pepsin is very rapidly inactivated at pH 8, while the pH of maximum stability of yeast polypeptidase is 9. In the majority of pH-activity curves published for enzymes, no differentiation is made between enzyme stability and enzyme activity, and it is certain that in many cases the factor limiting the activity at acid or alkaline pH values is enzyme destruction. If it is assumed that the neutral form of the enzyme is stable, that the acid and alkaline forms are unstable, that only one dissociation constant is involved, and that the half-life of the unstable form is small compared to the time of incubation, it may be shown that the pH-stability curve will be that of Figure 11. Variation in incubation time will not change the shape of the curve, but its position will change by one pH unit for every 10-fold increase in incubation time. This is illustrated in Figure 11. For alkaline inactivation, the curve is the mirror image of that for acid inactivation. Many enzymes follow this curve, but in many other cases, the process is more complex. An excellent example of complex pH behavior has been given by Kunitz and Northrop (1934). Figure 12, from their paper, gives data on the pH-stability characteristics of crystalline trypsin. Below pH 2, irreversible destruction occurs. At intermediate pH values, the active enzyme is in equilibrium with an inactive form which is hydrolyzed by the active enzyme. The rate of this reaction increases with pH, becoming very rapid in alkaline solution, where trypsin is most active. Between pH 12 and pH 13, however, the percentage of the total trypsin present as inactive form (substrate) is large, and the percentage present as active form (enzyme) is very small, hence the rate of inactivation decreases with increasing pH in this range. Above pH 13, irreversible alkaline inactivation occurs.

In the case of other enzymes, the pH stability behavior may be complicated by the presence of other enzymes which destroy the enzyme under study. The effects of pH on the stability and activity of the destroying enzyme then become of importance.

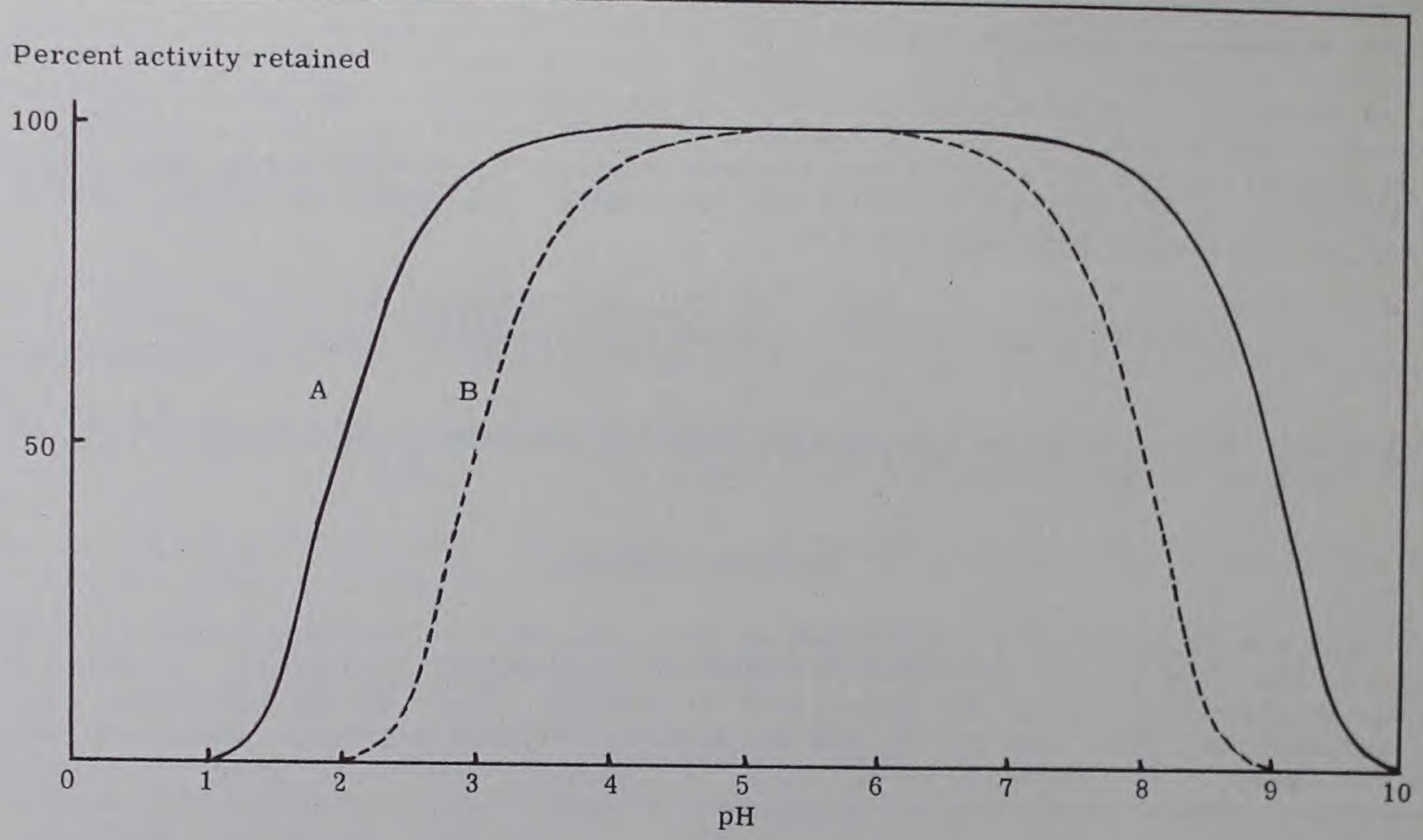


Figure 11

Hypothetical pH-stability curves for an enzyme unstable in both acid and alkaline solution. Curve B is for ten times the incubation time of curve A.

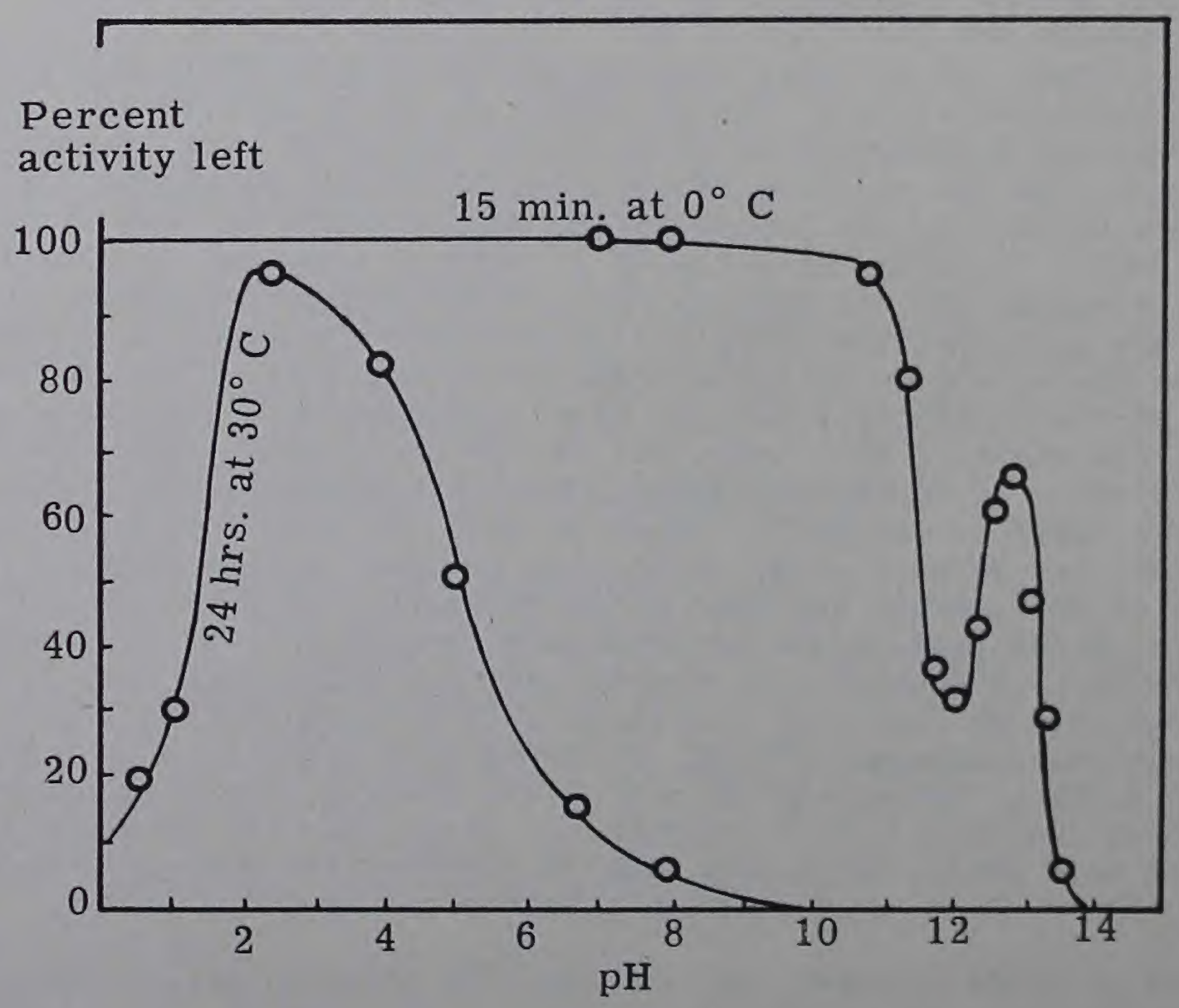


Figure 12

Effect of pH on stability of crystalline trypsin. (Kunitz and Northrop, 1934).

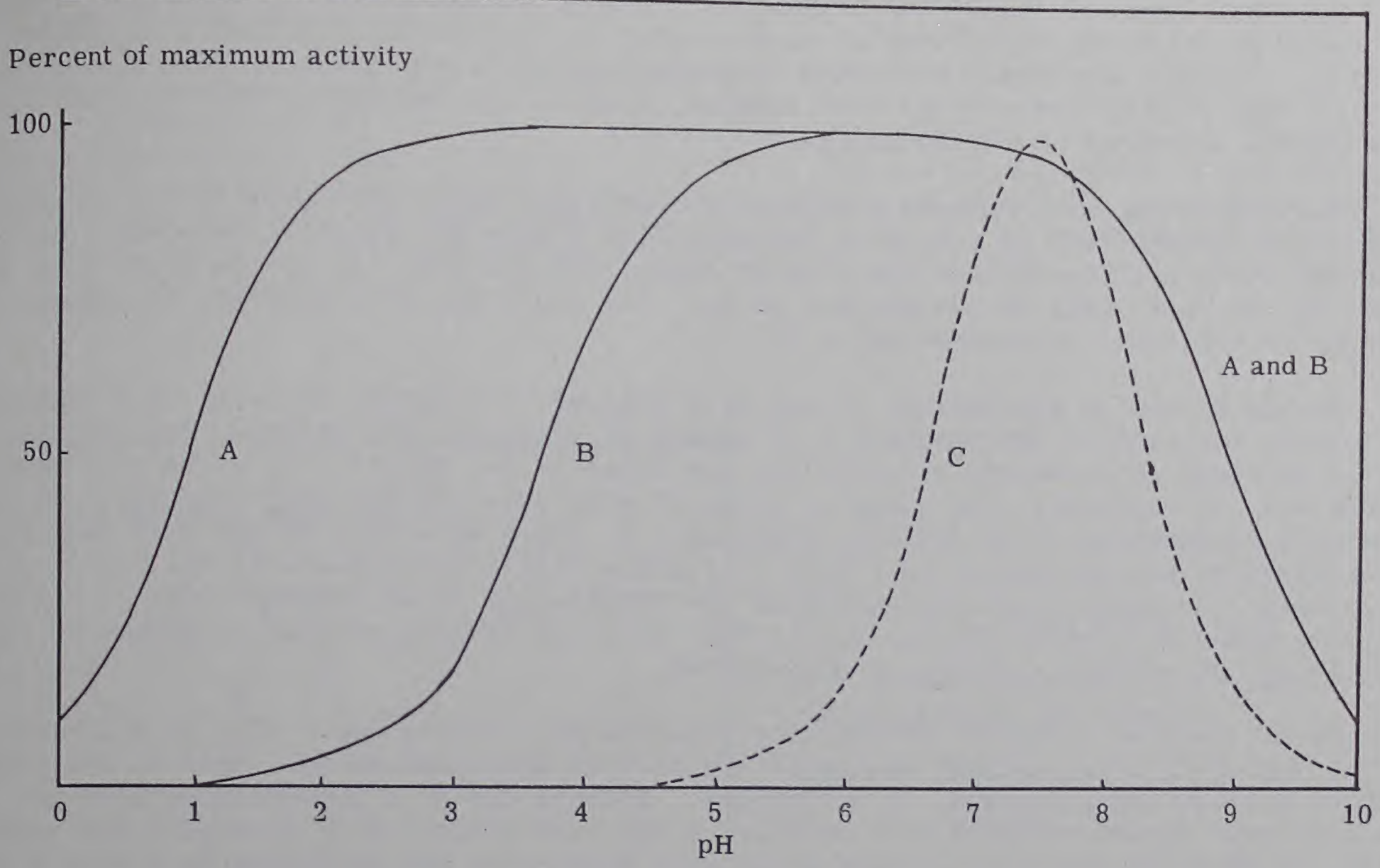


Figure 13

Hypothetical pH-activity curves for an enzyme limited at acid pH values by ionic form of the substrate, and at alkaline pH values by the ionic form of the enzyme. The enzyme is assumed to have a pK of 9.0 in all cases.

Curve A: Substrate pK = 4; K_s = 0.001
Curve B: Substrate pK = 4; K_s = 1.0
Curve C: Substrate pK = 11; K_s = 0.001

The effect of pH on enzyme activity (as distinguished from enzyme stability) may be occasioned by the effect of pH on the enzyme itself, on the substrate, or on activators or coenzymes. For illustration of the effect of pH on the enzyme itself, let us consider the simple case of an enzyme having an acidic dissociation constant (pK_a = x). If only the salt form of the enzyme is catalytically active, half-maximal activity will occur pH = x. The pH-activity curve will have the shape of the acidic arm of the curve of Figure 13A or 13B. This is the regular curve obtained by plotting per cent neutralization of any weak acid against pH.

The reversible combination of enzyme and substrate may be affected by pH independently of the reversible decomposition of the enzyme-substrate complex into product and enzyme (Van Slyke and Zacharias, 1914). Its ability to combine with coenzyme may also be affected by pH. Therefore, the "inactive form" of the enzyme, in the sense of the previous paragraph, may be inactive because of the loss of any one of a number of its properties. If effect of pH is on the velocity of combination with substrate, the apparent K_s will change with pH. Another manner in which pH may affect the activity of an enzyme is by its effect on the equilibrium between active enzyme and reversibly denatured enzyme. As was mentioned above, the equilibrium between trypsin and reversibly denatured trypsin is a function of pH, the pH at which half of the enzyme is in the denatured form being approximately 11.3 (Kunitz and Northrop, 1934).

Coenzymes and activators may, of course, be destroyed at extreme pH values, such destruction being observed as apparent enzyme destruction. If the coenzyme has a dissocia-

tion constant, and only one form is catalytically active, one arm of the pH activity curve will have the shape of Figure 13A or 13B, if coenzyme concentration is limiting. Cases are known in which the solubility of an activator is the determining factor in enzyme activity. Leucyl peptidase, activated by magnesium, decreases abruptly in activity above pH 9.2. The loss of activity is due (Johnson, Johnson and Peterson, 1936) to precipitation of the activator as the hydroxide.

Enzymes acting on a substrate with acidic or basic properties usually act on only one form of the substrate. If substrate concentration limits the reaction velocity, the pH-activity curve will again have the form of Figure 13A and 13B. It can be shown that the shape of the curve will be independent of K_s. Its position, will however, be changed with changes in substrate concentration or K_s.

In the foregoing discussion, it has been assumed that enzyme activity is determined as initial velocity of the reaction. If amount of reaction (for example, per cent hydrolysis) is taken as a measure of activity, the shape of the pH-activity curve will depend on the time of reaction. For example, since no more than 100 per cent conversion of the substrate is possible, a pH-activity experiment in which more than 90 per cent conversion is obtained at the optimum pH is likely to yield a fictitious plateau in the region of the pH optimum. In cases where the course of the reaction is first order at some pH values and zero order at others, the assumption that activity is proportional to amount of reaction at all pH values is obviously unjustified.

In the case of an actual enzyme, the observed pH-activity curve will be the resultant of the operation of an unknown number of the factors mentioned above. Many of the actual factors involved may, however, be determined. Effects due to a dissociation constant of the substrate may be detected most readily, since such dissociation constants are usually known. Effects on velocity of combination with substrates are reflected in a rise of apparent K_s. If the factor limiting enzyme activity at high pH values comes into operation at pH values greatly above the pH value in the acid region at which maximal activity is reached, the pH-activity curve will exhibit a plateau. If, however, limitation of the activity on the alkaline side occurs at a lower pH, a sharp pH optimum will result.

An example of the application of some of the above considerations to one actual case may be given. Data on the effect of pH on the hydrolysis of triglycine by intestinal aminopolypeptidase has been given by Johnson, Johnson and Peterson (1936). In the first two columns of Table 3, their data on the effect of pH on per cent hydrolysis of the substrate are reproduced. Since the reaction is first order (K_s large compared to substrate concentration), a figure proportional to initial velocity may be obtained by calculating $\log \frac{a}{a - x}$. This figure, given in the third column of Table 3, is proportional to the product of the active enzyme concentration and the active substrate concentration, expressed in arbitrary units. Since this enzyme hydrolyzes peptides at pH values where only

TABLE III

Aminopolypeptidase pH Relations

pH	Per cent hydrolysis	$\log \frac{a}{a - x}$ (= E x S)	Fraction active substrate (= S)	$\frac{E \times S}{S}$ (= active enzyme)
6.28	8	0.037	0.982	0.038
7.07	32	0.168	0.895	0.188
7.67	58	0.377	0.680	0.555
8.16	63	0.432	0.410	1.055
8.60	45	0.260	0.201	1.294
9.05	21	0.103	0.082	1.260
10.55	1	0.004	0.003	---

very small concentrations of peptide anion are present, it is reasonable to assume that it attacks the zwitterion form of the substrate. The pK_a of triglycine is very close to 8.0. The fraction of the total substrate in available form is therefore readily calculated, and is given in column 4. Since the figures in column 3 are proportional to substrate times enzyme, and in column 4 to substrate, their quotient should give the concentration of available enzyme at various pH values. This quotient is tabulated in column 5. A plot of the data of column 5 is shown in Figure 14, curve A. The points are those of the table, and the solid line is the theoretical line for a substance of pK = 8.0. A good fit was obtained by assuming that the total enzyme concentration was 1.77, expressed in the arbitrary units of column 5. It will be noted that the points at pH 8.6, and pH 9.05 fall be-

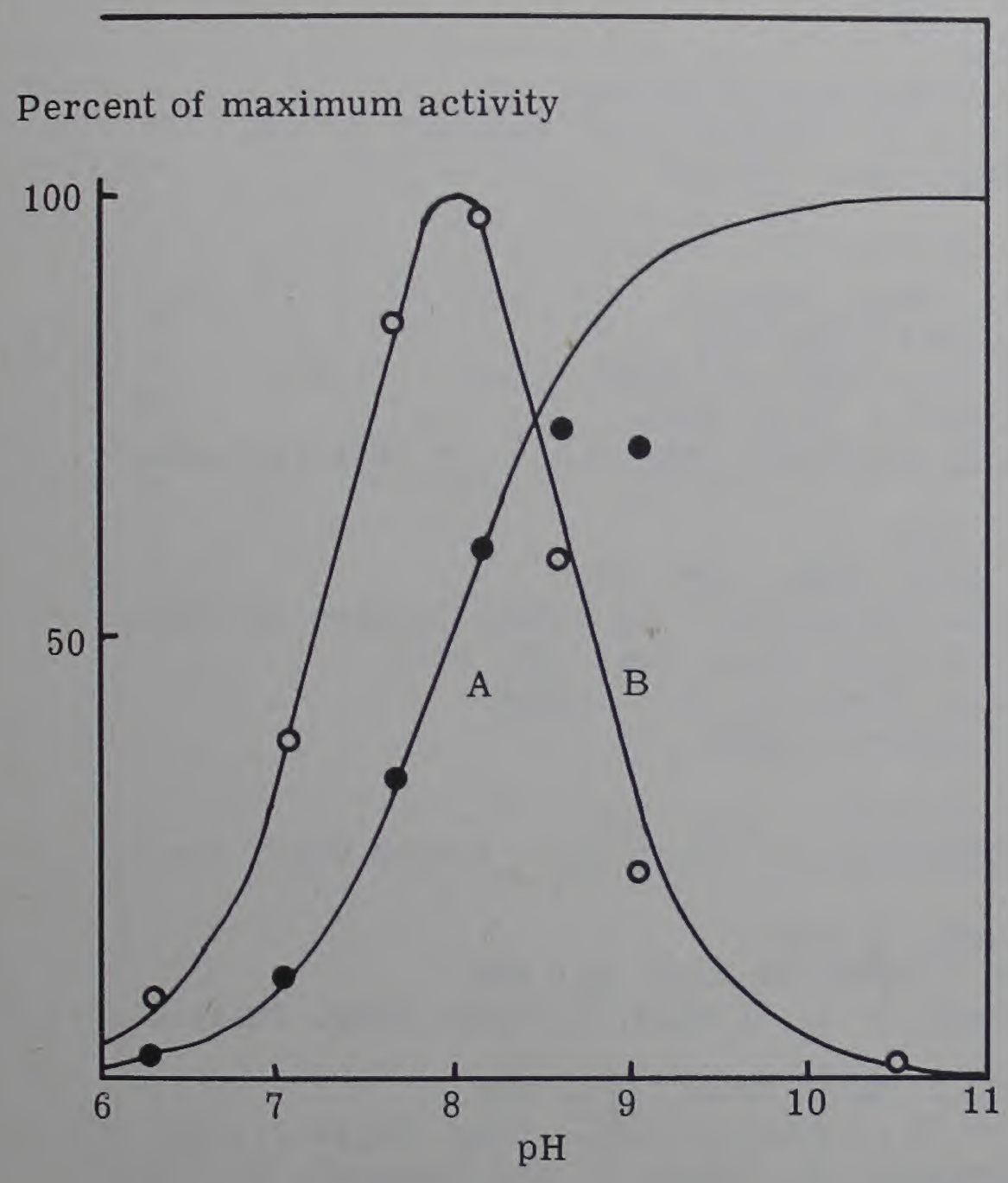


Figure 14

pH Relationships for intestinal aminopolypeptidase.

Curve A: The points are the observed concentrations of active enzyme as calculated in Table 1, the solid line is the theoretical curve for a substance of pK 8.

Curve B: The points are observed enzyme activities, the solid line represents activity as calculated on the assumptions given in the text.

low the theoretical, possibly because of enzyme inactivation, which was not controlled. The solid line of curve B, Figure 14 is a theoretical curve for reaction velocity based on the following assumptions:

1. Only the zwitterion form of the substrate is attacked.
2. The enzyme has a dissociation constant of 10^{-8}, only the acidic form being active.
3. The total enzyme concentration is 1.77 arbitrary units.

The points on the curve are the observed activities, from column 3. It will be noted that the agreement is good, indicating that the assumptions made are consistent with the observed behavior of the enzyme.

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Chapter III

OXIDATION-REDUCTION POTENTIALS

by

MARVIN J. JOHNSON

Professor of Biochemistry

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THE HYDROGEN ELECTRODE

An inert metallic electrode, like all other pieces of metal, contains "free" electrons, that is, electrons capable of moving freely within the metal. A concerted movement of such electrons in one direction constitutes an electric current. An electric potential may be crudely described as an electron pressure tending to cause electron movement.

If an inert metallic electrode (by "inert" is meant chemically inert under the conditions of the experiment; noble metals are generally used) is immersed in an aqueous solution, electrons may leave the electrode and enter the solution, or they may leave the solution and enter the electrode. An aqueous solution does not contain free electrons in the sense that metals do. Therefore, electrons entering the solution from the electrode can do so only by combination with a component of the solution, and electrons entering the electrode from the solution must arise from decomposition of a component of the solution. A hydrogen ion, for example, can combine with an electron, becoming a hydrogen atom, or a hydrogen atom may decompose into an electron and a hydrogen ion.

$$1/2\ H_2 \rightleftharpoons H^+ + e \qquad (1)$$

$$K = \frac{(H^+)\cdot(e)}{\sqrt{(H_2)}}$$

If an inert electrode is immersed in a solution containing both hydrogen ions and dissolved hydrogen gas, and if an agent (platinum black, hydrogenase, or other catalyst) is present capable of activating the hydrogen, reaction (1) will take place until equilibrium is reached, that is, until electrons enter and leave the electrode at equal rates. If the hydrogen ion concentration of the solution is then increased, reaction (1) will be driven toward the left, and more electrons will leave the electrode, causing it to assume a more positive potential. If, on the other hand, the hydrogen pressure is increased, the reaction will be driven toward the right, and more electrons will enter the electrode, causing it to assume a more negative potential. The potential of the electrode will, in either case, establish itself at a new value corresponding to the new equilibrium point of reaction (1). Obviously, therefore, the potential of the electrode is a function of the ratio $\frac{(H^+)}{\sqrt{(H_2)}}$.

The potential existing between an electrode and the solution in which it is immersed cannot be measured, since such a measurement could only be made by inserting another electrode into the solution, which, of course, would only introduce another electrode potential. We can, therefore, speak only of the difference in potential of two electrodes in the same solution. In the present sense, two different solutions connected by a salt bridge constitute a single solution. If we arbitrarily assign a value of zero to some specific electrode, we can refer the potentials of all other electrodes to it, and express their potentials numerically. The normal hydrogen electrode has been universally adopted as such a standard reference electrode. The normal hydrogen electrode consists of an inert metal electrode in a solution normal with respect to hydrogen ions (pH = 0), and in equilibrium with hydrogen gas at a pressure of one atmosphere. A hydrogen-activating catalyst must be present. Such an electrode has, by definition, a potential of zero at all temperatures.

DERIVATION OF THE ELECTRODE EQUATION

In order to understand the quantitative relationships connecting hydrogen pressure, hydrogen ion concentration, and electrical potential, let us consider a cell consisting of two hydrogen electrodes. A cell is composed of two electrodes (also often called "half-

cells") immersed in the same solution. The cell of Figure 1 consists of two electrodes in two vessels, A and B, joined by a liquid bridge sufficiently small to render diffusion negligible. Electrode A is a normal hydrogen electrode. In vessel B, $(H_2)_B = 1.0$, but the hydrogen ion concentration has some value $(H^+)_B$ which we will assume is less than unity; that is, vessel B has the higher pH.

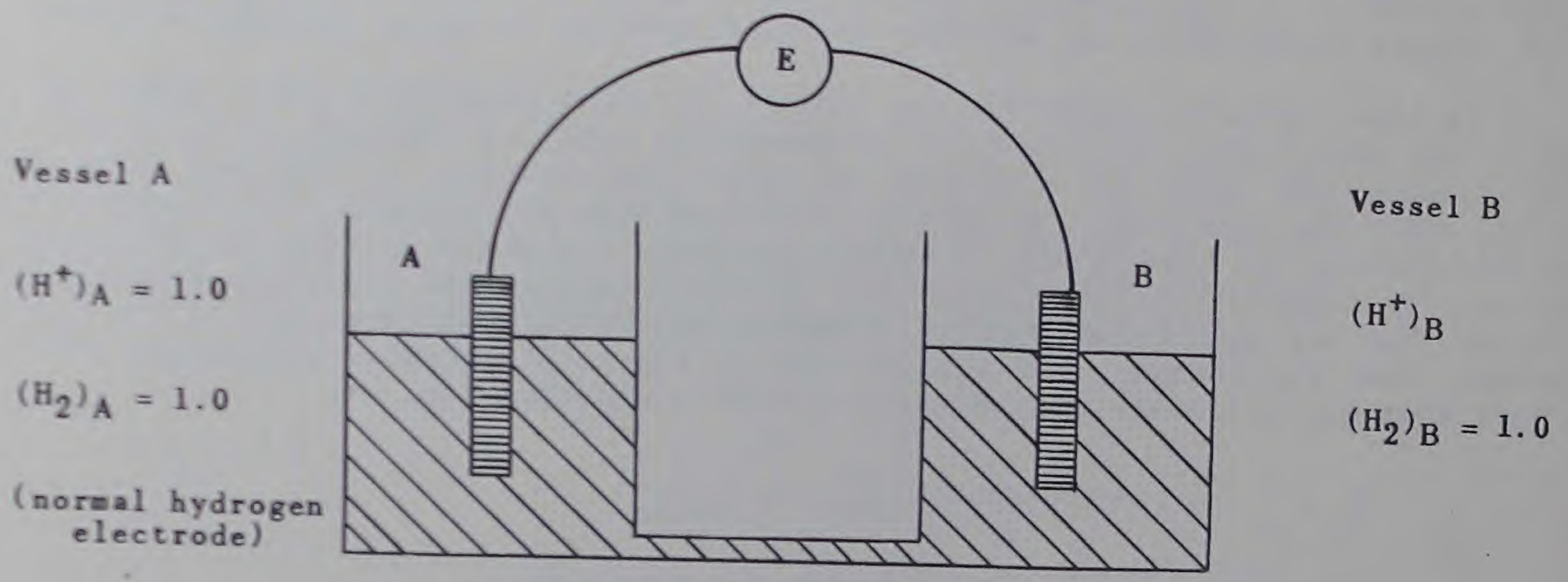


Figure 1

If the two electrodes are connected by a conducting wire, electrons will move through this conductor from vessel B to vessel A, since in vessel A equation (1) will be driven farther toward the right than in vessel B, because of the higher hydrogen ion concentration in vessel A. We will thus have, at electrode B the reaction

$1/2\ H_2 \longrightarrow H^+ + 2\ \underline{e}$ taking place, while at electrode A the reaction

$H^+ + \underline{e} \longrightarrow 1/2\ H_2$ will proceed. (Of course, in order to maintain the balance between positive and negative ions in each of the solutions, negative ions, e.g., Cl^- ions, will at the same time migrate through the liquid bridge from vessel A to vessel B). Thus we have effectively a transfer of H^+ ions from vessel A, where the H^+ concentration is high, to vessel B, where it is lower, since every time a H^+ ion is destroyed in vessel A, one is created in vessel B. This process will continue until the hydrogen ion concentration in both vessels is the same.

If, instead of connecting the two electrodes by means of a conductor, we connect them by means of a device which will measure the tendency for electrons to flow without actually permitting them to flow (an electrometer is such an instrument) we will observe that electrode B has a more negative potential than electrode A, since electrons will be entering electrode B and leaving electrode A. This potential difference between the two electrodes can not only be measured, but it may be calculated, and moreover calculated values have been found to check measured values.

The calculation is based upon the fact that in dilute solutions, ions obey the gas laws. It is well known that the work necessary to compress a volume of gas by a small amount is equal to the volume of the gas times its small change in pressure.

$$dW = VdP$$

or

$$W = \int_{P_2}^{P_1} VdP$$

Since, for one mole of gas PV = RT (where R is the molar gas constant)

$$W = \int_{P_2}^{P_1} RT\ 1/P\ dP$$

$$W = RT\ \ln P_1/P_2.$$

Since ions obey the gas laws, the work done in transferring one mole of hydrogen ions from one concentration to another is

$$W = RT\ \ln \frac{C_1}{C_2}$$

or, in the case of Figure 1, the work done by 1 mole of H^+ ions in "expanding" from vessel A to vessel B is

$$W = RT\ \ln \frac{(H^+)_B}{(H^+)_A}.$$

Since $(H^+)_A = 1$,

$$W = RT\ \ln (H^+)_B.$$

If we wish to express the above equation in electrical units, the work W must be expressed in joules (volt coulombs). Since the number of coulombs corresponding to the transfer of 1 mole of ions is 96,500 (1 faraday (F) is 96,500 coulombs), the electrical work corresponding to the transfer of 1 mole of ions is EF, where F is the faraday and E is the potential, in volts, between the two electrodes. Therefore

$$EF = RT\ \ln (H^+)_B, \quad \text{or} \quad E = \frac{RT}{F}\ \ln (H^+)_B. \qquad (2)$$

That is, the potential of electrode B, when measured against electrode A, which has by definition zero potential, is equal to a known constant times the logarithm of the hydrogen ion concentration in vessel B. Inserting numerical values for the known constants, assuming 30° C., and changing to common logarithms, we have

$$E = \frac{\overset{(R)}{8.315} \times \overset{(T)}{303.1} \times \overset{(\ln 10)}{2.3026}}{\underset{(F)}{96{,}500}}\ \log (H^+)_B$$

$$E = 0.06\ \log (H^+)_B, \text{ or}$$

$$-E = 0.06\ pH_B.$$

That is, at 30°, the negative potential of electrode B will be 0.06 times its pH. This relation really holds in practice, and is used to determine pH. What we have been describing is really nothing more than a pH determination by means of the hydrogen electrode.

If, in Figure 1, the hydrogen ion concentration of vessel B had been held at unity, and the hydrogen pressure had been set at some value less than 1 atmosphere there would have occurred a transfer of electrons from vessel A to vessel B. At electrode A the reaction

$$1/2\ H_2 \longrightarrow H^+ + \underline{e}$$

would take place. The electrons yielded by the reaction would flow through the wire to electrode B, where the opposite reaction

$$H^+ + \underline{e} \longrightarrow 1/2\ H_2$$

would take place. Thus we would have effectively a transfer of hydrogen gas from vessel A to vessel B, which would continue until equilibrium is reached. By a consideration of the work done in the transfer of this hydrogen, we can derive, in a manner similar to that just described for hydrogen ions, an expression for the relation between hydrogen pressure difference and potential difference. The expression which we obtain for the case under consideration, where the electrode solution has unit hydrogen ion concentration is

$$-E = \frac{RT}{F} \ln \sqrt{(H_2)_B} \qquad \text{or}$$

$$-E = \frac{RT}{2F} \ln (H_2)_B \qquad (3)$$

or, at 30°,

$$-E = 0.03 \log (H_2)_B.$$

Thus the negative potential of an electrode at zero pH is equal, at 30°, to 0.03 times the log of the hydrogen pressure in equilibrium with it.

By combining equations (2) and (3), we arrive at an expression completely describing the potential of an inert electrode in any dilute aqueous solution whatever.

$$E = \frac{RT}{F} \ln \frac{(H^+)}{\sqrt{(H_2)}} \qquad \text{or}$$

$$E = \frac{RT}{F} \ln H^+ - \frac{RT}{2F} \ln (H_2) \qquad (4)$$

or, at 30°,

$$E = 0.03 \log \frac{1}{(H_2)} - 0.06\ pH. \qquad (4a)$$

Equation (4) is the fundamental electrode equation. We can, by its use, determine either pH or hydrogen pressure, providing the other is known. By holding (H_2) at some known constant value, we are able to make potentiometric pH determinations. On the other hand, if the pH of a solution is known, we can determine from the potential what the hydrogen pressure in the solution is.

OXIDATION-REDUCTION POTENTIALS*

Let us consider a solution in which the hydrogen pressure arises from a reaction in the solution capable of liberating hydrogen. An example of such a solution is one contain-

*In the following presentation the relations governing oxidation-reduction potentials will be derived by expressing the reducing intensity of a solution in terms of the pressure of hydrogen gas with which it would be in equilibrium. This formulation has been chosen as preferable for students in biochemistry for a number of reasons. Students are always able to formulate reduction reactions in which hydrogen is the reductant, but are often confused when required to write chemical equations involving the reduction of organic compounds by electrons. Moreover, the concept of the hydrogen pressure in equilibrium with a system is more easily grasped by students lacking adequate electrochemical background than is the apparently more abstract concept of the "electron pressure" of the system.
The hydrogen formulation lends itself readily to a presentation of oxidation-reduction potentials in terms of the hydrogen electrode. Once the effect of a change in hydrogen pressure on the hydrogen electrode is understood, all other oxidation-reduction electrodes become explicable as modified hydrogen electrodes in which the hydrogen pressure present is that in equilibrium with the system.

ing formic acid (or formate ion) and hydrogenlyase, which is an enzyme catalyzing the reversible decomposition of formic acid into carbon dioxide and hydrogen. At pH values low enough that neither formic acid nor carbon dioxide are ionized, the reaction is

$$HCOOH \rightleftharpoons CO_2 + H_2$$

In the solution, hydrogen gas will be liberated, and dissolve until its pressure reaches the point corresponding to the equilibrium point of the reaction. An inert electrode immersed in the solution can be used to measure this hydrogen pressure. The measurement would consist merely of a determination of the potential and substitution of the potential in equation (4). It should be emphasized that addition of a hydrogen-activating catalyst would not be necessary, since (1) the hydrogen liberated by the reaction is active until it is transformed into molecular hydrogen, and (2) the hydrogenlyase system is capable of reducing carbon dioxide with molecular hydrogen, and hence capable of activating molecular hydrogen.

Some organic compounds, such as methylene blue, will react with activated hydrogen without the intermediation of a dehydrogenase. Hydrogen, in the presence of a hydrogen-activating catalyst, will reduce methylene blue vigorously, even at extremely low hydrogen pressures. It is obvious, however, that the reaction

$$MB + H_2 \rightleftharpoons MBH_2$$

has a definite equilibrium point, and that at sufficiently low hydrogen pressures, methylene blue would not be completely reduced. It is equally true that a solution of leucomethylene blue behaves like a solution possessing an extremely small but definite pressure of active hydrogen. This hydrogen is capable of reacting with an inert electrode in the solution, or of reducing another autoxidizable compound (for instance a quinone) added to the solution. The hydrogen pressure of a methylene blue solution may also be measured by means of an electrode.

It is apparent that, as a generalization of the above, a solution of any autoxidizable system (including systems made autoxidizable by the presence of suitable enzymes) behaves toward an electrode as though there were present the pressure of hydrogen which would be in equilibrium with the system. As a corollary, it is apparent that the reducing intensity of any solution may be expressed as the pressure of hydrogen with which it is in equilibrium, or, alternatively, as the corresponding electrode potential.

It may be seen from inspection of equation (4) that, of two systems at the same pH, the system with the more negative potential will be the more active reducing agent, that is, will be in equilibrium with the higher pressure of hydrogen. In order to obtain a more quantitative picture, however, it is necessary to take into account the concentrations of the constituents of the system.

DERIVATION OF OXIDATION-REDUCTION POTENTIAL EQUATIONS

In the reaction

$$\text{succinate} \rightleftharpoons \text{fumarate} + H_2$$

$$K = \frac{(\text{fumarate})(H_2)}{(\text{succinate})}, \text{ and}$$

$$(H_2) = \frac{K\,(\text{succinate})}{(\text{fumarate})}.$$

The hydrogen pressure in equilibrium with the system therefore depends only on the ratio of succinate to fumarate. The potential of a solution containing succinate and fumarate will therefore be the potential of a hydrogen electrode the hydrogen pressure of which is

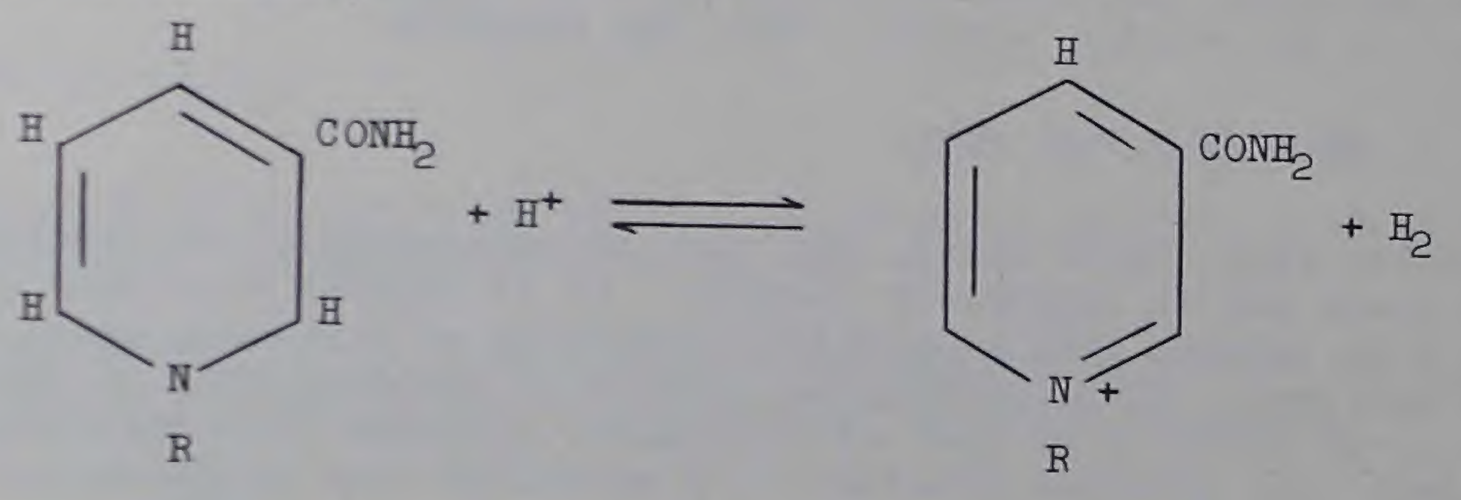
that in equilibrium with the succinate-fumarate ratio of the solution. The potential will vary with pH in the same manner as any other hydrogen electrode (see equation 4a).

If, however, the system is such that an acid or base is produced by the oxidation or reduction, another factor must be taken into account. In such a system as

$$CH_3CHO \rightleftharpoons H_2 + CH_3\,COO^- + H^+,$$

$$(H_2) = \frac{K\,(CH_3CHO)}{(CH_3COO^-)(H^+)}\ ;\ \text{therefore}$$

the hydrogen pressure in equilibrium with the system depends not only on the ratio of oxidant to reductant, but also on the hydrogen ion concentration. The potential of the system will therefore vary with pH not only according to the relation of equation (4), but also in an additional manner due to the variation of the hydrogen pressure itself with pH. In the acetaldehydeacetate system, a hydrogen ion is <u>produced</u> in the oxidation reaction. In other systems, hydrogen ions may <u>disappear</u> in the oxidation reaction. This occurs, for example when DPN or TPN is oxidized,



since a weakly basic nitrogen atom is oxidized to a strongly basic quaternary nitrogen.

Let us therefore express the general case as follows:

$$\text{reductant} + aH^+ \rightleftharpoons \text{oxidant} + n/2\ H_2$$

where "a", the number of hydrogen ions disappearing in the oxidation reaction, may be positive, negative, or zero. Since, in accepted usage, "n" is the number of electrons or hydrogen atoms involved in the reaction, the number of hydrogen molecules involved becomes n/2. Applying the mass law to this general equation, we obtain

$$(H_2) = \left(\frac{K\,(red)(H^+)^a}{(ox)}\right)^{2/n}$$

as the quantitative expression for the hydrogen pressure in equilibrium with the system. In order to obtain a quantitative expression for the potential of the system, it is necessary only to substitute the above expression for (H_2) in equation (4).

$$E = \frac{RT}{F}\ \ln H^+ - \frac{RT}{2F}\ \ln \left(\frac{K\,(red)\,(H^+)^a}{(ox)}\right)^{2/n}$$

This simplifies algebraically to

$$E = \frac{RT}{nF}\ \ln\ \frac{(ox)}{(red)}\ - \frac{RT}{nF}\ \ln K + \frac{n - a}{n}\ \frac{RT}{F}\ \ln H^+.$$

This equation gives the potential of an inert electrode in any aqueous solution of an autoxidizable oxidoreduction system. The constant K has a characteristic value for

each system. The term E is often used in place of the cumbersome expression - $\frac{RT}{nF}$ ln K. The general equation then becomes

$$E = E + \frac{RT}{nF} \ln \frac{(ox)}{(red)} + \frac{n - a}{n} \frac{RT}{F} \ln H^+. \qquad (5)$$

At 30° C., the equation becomes

$$E = \tilde{E} + \frac{0.06}{n} \log \frac{(ox)}{(red)} + \frac{0.06\ (a-n)}{n} pH \qquad (5a)$$

where E is a constant characteristic of the system, "a" is the number of hydrogen ions formed in the reduction reaction, and n the number of hydrogen atoms required in the reduction. Equation (5) is a general equation describing the behavior of an electrode in any oxidoreduction system. The constant $\tilde{E}$ can be readily determined experimentally by a potential measurement in a solution for which both pH and the ratio of oxidant to reductant are known. In the half-reduced system, the second term on the right becomes zero, and the pH term should also become zero when the pH is zero. At pH zero the potential of the half-reduced system should therefore be equal to $\tilde{E}$. In many systems, however, the constant "a" is not the same at all pH values. For example, at low pH values, the oxidation of acetaldehyde to acetic acid does not liberate a hydrogen ion since acetic acid is not ionized at low pH values. The expression $\tilde{E}$, therefore, might be described as the potential the half-reduced system would have at pH zero if the characteristics of the system did not change between pH zero and the pH at which $\tilde{E}$ was determined. $\tilde{E}$ and "a" do not change with pH in pH regions remote from a dissociation constant of the oxidant or reductant.

It should be pointed out, however, that equation 5 holds at any pH if changes in the oxidant or reductant concentration due to dissociation, precipitation, or other causes are taken into account. Thus the $\tilde{E}$ value for the acetaldehyde-acetate system, as determined at pH 7, holds to pH zero if the oxidant concentration in equation (5) is considered to be the acetate ion concentration at the pH value in question. For example, at pH zero, the acetate ion concentration is 0.000018 times the acetic acid concentration, so that

$$\frac{(ox)}{(red)} = \frac{(acetate^-)}{(acetald.)} = \frac{0.000018\ (acetic\ acid)}{(acetald.)}.$$

The term used to express the actual value of the potential of the half-reduced system at pH zero is E_o. When the system does not change in properties between pH zero and the pH range under investigation, E_o and $\tilde{E}$ are identical. The term E_o has the advantage of being a more real quantity than $\tilde{E}$, but has the disadvantage that it is difficult to calculate the potential of a system from the knowledge of E_o, the pH, and the ratio of oxidant to reductant.

Because of the complexity of the dependence of potential on pH, a simplification of equation (5) is often used. If a system is to be studied at only one pH value, the pH term of equation 5 has a constant value. Hence, a new constant, combining $\tilde{E}$ and the pH term, may be used. This constant is called E'_o.

$$E'_o = \tilde{E} + \frac{n - a}{n} \frac{RT}{F} \ln H^+$$

Equation (5), therefore, at constant pH, simplifies to

$$E = E'_o + \frac{RT}{nF} \ln \frac{(ox)}{(red)} \qquad (6)$$

or, at 30° C., to

$$E = E'_o + \frac{0.06}{n} \log \frac{(ox)}{(red)} \qquad (6a)$$

It is obvious that E_o' may be defined as the potential of the half-reduced system at the pH under consideration. It should be noted that a given value for E_o' holds only for a single pH value, while $\tilde{E}$ is constant throughout a range of pH values.

Table 1 illustrates by typical examples the pH relations existing in various types of oxidation-reduction systems. For determining the manner in which the potential of any system varies with pH, the following procedure may be followed:

1. Write a balanced equation representing the reduction of the oxidant by gaseous hydrogen.

2. Note the number of equivalents of acid produced (or base destroyed) in the reaction thus written. This number is a, and may be positive, zero, or negative. In determination of acid or base produced, count only acids or bases that are almost completely ionized in the pH range under consideration. Thus, CO_2 is not an acid at pH 3, is a monobasic acid at pH 8, and is a dibasic acid at pH 14. Acetic acid is an acid at pH 7 but not at pH 3. Ammonia is a base at pH 5 but not at pH 13. Hydrogen ion is acidic at any pH, and hydroxyl ion is basic at any pH.

3. Note the number of atoms of hydrogen used in the reduction reaction as written. This is n. It is always positive.

4. The increase in E_o' for a unit increase in pH is, at 30°

$$\frac{dE_o'}{dpH} = \frac{0.06\ (a-n)}{n}.$$

REACTIONS BETWEEN OXIDATION-REDUCTION SYSTEMS

We have seen that at a given pH, the electrode potential of a solution depends upon the theoretical hydrogen pressure of that solution. The potential is proportional to the logarithm of the reciprocal of the hydrogen pressure. If one solution is 0.03 volts more negative than another, the first solution has 10 times the hydrogen pressure of the second.

If two solutions, having the same pH, but different potentials, are mixed, reaction will take place. It is convenient to visualize this reaction as taking place because of the difference in hydrogen pressure between the two systems, hydrogen "expanding" from one system to the other until equilibrium is established. A better, if slightly less convenient, picture is that of electrons being transferred from one system to the other until the electron pressure of both systems is equal.

The reaction which takes place upon mixing of two oxidation-reduction systems will, of course, proceed until equilibrium is reached, that is, until both systems have arrived at the same potential. The ratio (ox)/(red) of one system will decrease, thus decreasing its potential. At the same time the (ox)/(red) ratio for the other system will increase, increasing its potential. This process will continue until both potentials are the same. An example will make this clear. Suppose, in an evacuated Thunberg tube, we have 1 ml. of a solution 0.001 M with respect to both malate and oxalacetate, containing a malate dehydrogenase system. In the side-arm we have 1 ml. of a solution 0.001 M with respect to both methylene blue and leuco methylene blue. Both solutions are at pH 7. Since both systems are half reduced, $E = E_o'$. The malate-oxalacetate system will have a potential of -0.102 volts, and the methylene blue system will have a potential of -0.011 volts. We now mix the two solutions. Since the malate system has the lower potential, it will begin to reduce the dye system. As the reaction proceeds, however, the ratio malate/oxalacetate decreases, thereby increasing the potential of this system. At the same time, the ratio MBH_2/MB increases, thereby decreasing the potential of this system. The reaction will continue until both systems have the same potential, that is, until equilibrium has been

TABLE I

pH Relations of Oxidation-Reduction Systems

n H atoms required for reduction	a H ions produced by reduction	Examples	Equation	Increase in E'_o per unit increase in pH (30°)	Increase in E per unit increase in $\log \frac{(ox)}{(red)}$
1	1	Cytochrome Fe^{++} - Fe^{+++}	$E = E + \frac{RT}{F} \ln \frac{(ox)}{(red)}$	volts 0.0	volts 0.06
2	0	Quinone-hydroquinone (below pH 8) Lactate-pyruvate (above pH 6) Yellow enzyme MB-MBH_2 (above pH 6)	$E = E + \frac{RT}{2F} \ln \frac{(ox)}{(red)} + \frac{RT}{F} \ln (H^+)$	-0.06	0.03
2	1	TPN; DPN (below pH 9)	$E = E + \frac{RT}{2F} \ln \frac{(ox)}{(red)} + \frac{RT}{2F} \ln (H^+)$	-0.03	0.03
2	1	Acetaldehyde-acetate (above pH 7) MB-MBH_2 (below pH 5)	$E = E + \frac{RT}{2F} \ln \frac{(ox)}{(red)} + \frac{3RT}{2F} \ln (H^{\ddagger})$	-0.09	0.03
2	2	Sn^{++} - Sn^{++++}	$E = E + \frac{RT}{2F} \ln \frac{(ox)}{(red)}$	0.0	0.03
n	a		$E = E + \frac{RT}{nF} \ln \frac{(ox)}{(red)} + \frac{(n-a).RT}{nF} \ln (H^+)$	$\frac{0.06\ (a-n)}{n}$	$\frac{0.06}{n}$

reached. Then,

$$-0.102 = 0.03 \log \frac{(oxal)}{(mal)} = 0.011 + 0.03 \log \frac{(MB)}{(MBH_2)} \quad \text{or}$$

$$0.03 \log \frac{(oxal)}{(mal)} = 0.113 + 0.03 \log \frac{(MB)}{(MBH_2)}.$$

Since, in our simple case,

$$(MB) = (mal) \text{ and } (MBH_2) = (oxal)$$

at all stages of the reduction process,

$$0.06 \log \frac{(oxal)}{(mal)} = 0.113, \text{ and}$$

$$\frac{(oxal)}{(mal)} = \frac{(MBH_2)}{(MB)} = 76.4.$$

This $(MBH_2)/(MB)$ ratio corresponds to 98.6% reduction of the dye. That is, at equilibrium, an appreciable portion of the dye would remain unreduced.

Another example might be of interest. How much pyruvate must be added to 0.01 M lactate (at pH 7) to cause sufficient "inhibition" that coenzyme I can be reduced by the lactate to an extent of only 0.1%? (The necessary enzymes are assumed to be present.)

Lactate system

$$E = -0.180 + 0.03 \log \frac{(pyr)}{(lact)}$$

Coenzyme I system

$$E = -0.254 + 0.03 \log \frac{(Co)}{(CoH_2)}$$

Let the desired pyruvate/lactate ratio be x.

$$-0.180 + 0.03 \log x = -0.254 + 0.03 \log \frac{999}{1}$$

$$0.03 \log x = -0.074 + 0.09$$

$$0.03 \log x = +0.026$$

$$\log x = 0.867$$

$$x = 7.36$$

$$\frac{(pyr)}{0.01} = 7.36$$

$$(pyr) = 0.0736$$

That is, the presence of 0.0736 M pyruvate would cause the desired degree of inhibition.

ENERGY RELATIONS

The potential difference between two reacting systems in an oxidoreduction bears a simple relationship to the free energy of the reaction. (We are considering now not the difference in E'_o values, but the actual potential difference, which will, of course, depend on the concentration of the reactants.) If the potential difference is ΔE, then the electrical work capable of being done by the formation of 1 mole of product is of course

$$-\Delta \underline{F} = nF \Delta E$$

Where $\Delta \underline{F}$ is the change in energy content of the system, n the number of electrons involved, and F the faraday. $\underline{F}$ is here expressed in joules. A calorie is equal to 4.18 joules. A foot-pound is 1.36 joules. Let us assume that in a living cell, lactate is being dehydrogenated to pyruvate, the hydrogen being transferred, through coenzymes or any other carriers, to cytochrome C. Let us further assume that the cell maintains the cytochrome in a half-reduced state, and that lactate and pyruvate are present in equal concentrations. We need not know anything about the carrier system in order to calculate the free energy change of the complete reaction. The lactate-pyruvate system has an E'_o of -0.180 at pH 7. The corresponding value for the cytochrome C system is + 0.262°. Since both systems are in the half-reduced state, $E = E'_o$, and

$$\Delta E = 0.444 \text{ volts}$$

$$-\Delta \underline{F} = 2 \times 96{,}500 \times 0.444 = 85{,}500 \text{ joules} = 20{,}500 \text{ cal.}$$

That is, the energy liberated by the reduction of one mole of cytochrome by one mole of lactate under the above conditions would, if it could be completely utilized, burn a 100 wall bulb for 14 minutes or enable a 160-pound man to climb 30 flights of stairs.

Another important relationship between potential and energy change may be derived from the equation:

$$-\Delta \underline{F} = nF \Delta E$$

If ΔE is the difference in potential between some system and the hydrogen system (hydrogen electrode), $-\Delta \underline{F}$ becomes the free energy of the reaction of hydrogen gas with the system. Thus the standard free energy of reduction of a system is equal to $nF\ (E'_o + 0.06\ pH)$, since (-0.06 pH) is the potential of the hydrogen electrode.

For the reaction

$$H_2 + 1/2\ O_2 \rightleftharpoons H_2O$$

the standard free energy is

$$-\Delta \underline{F} = 56{,}560 \text{ calories} = 237{,}000 \text{ joules.}$$

To find the potential of the oxygen system (O_2 at 1 atmosphere in the presence of an oxygen-activating enzyme, if there be such) we may use the expression

$$-\Delta \underline{F} = nF\ (E'_o + 0.06\ pH)$$

At pH 7 and 30°

$$237{,}000 = 2 \times 96{,}000\ (E'_o + 0.420)$$

$$E'_o = 0.81$$

At pH 7, therefore, the potential of the oxygen system in aqueous solution, where reductant concentration (H_2O) is always unity, is

$$E = E'_0 + \frac{RT}{4F} \ln (O_2)$$

where (O_2) is the oxygen pressure in atmospheres. The potential corresponding to 1/5 atmosphere of oxygen is therefore 0.81 -0.015 log 5, or 0.80 volts.

One more example of energy calculation may be given.

In a previous example we found that the energy liberated when lactate reduced cytochrome C was 20,500 calories when both systems were half-reduced. Let us now see how many additional calories would be liberated if the reduced cytochrome reacts with atmospheric oxygen.

$$\Delta E = 0.80 - 0.444 = 0.356$$

$$-\Delta F = nF \Delta E$$

$$-\Delta F = 2 \times 96{,}500 \times 0.356 = 68{,}600 \text{ joules} \quad \text{or} \quad 16{,}500 \text{ calories.}$$

Thus almost as much energy is available from the step from cytochrome to oxygen as is available in the step from lactate to cytochrome. The energy in either case is, of course, not available to the cell unless coupling mechanisms exist.

Chapter IV

TABLE OF OXIDATION-REDUCTION POTENTIALS

by

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and

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INTRODUCTORY COMMENT

SCOPE

The table of oxidation-reduction potentials which forms the bulk of this chapter represents an attempt to compile a comprehensive list of systems of interest to workers in the biological sciences. No attempt has been made to critically evaluate the results of the many determinations which have been quoted from the original literature. The values for the inorganic systems quoted from Latimer and Hildebrand, and those taken from the review by Kalckar* represent the considered judgement of those authors. Many of the biologically important systems have been investigated repeatedly during the last thirty years. The diverse values obtained by successive workers can be attributed to the failure of some of them to overcome the inherent difficulties presented by many of these systems. In these cases the authors have used the results of the investigation which seemed to them to have the soundest basis. Two or more values, obtained by different investigators, have been listed for several systems whose potentials are controversial.

NOMENCLATURE

The listing of both the oxidized and reduced components of every system would have resulted in the unnecessary repetition of many long chemical names identical except for one or two syllables. Where the designation of both components is necessary, the reduced form has been written first, and the system will be found in the alphabetical list under this heading. When only one component is named, the form which is best known has been selected. Examples of systems designated by a single component are the quinones, the flavins, and the many dyes which are used as redox indicators.

THE ALPHABETICAL LIST

In order to facilitate the location of any desired system in the table, an alphabetical list precedes the main table. No rigorous plan has been followed in the alphabeting. Rather, an attempt has been made to put each compound in the place where, in the authors judgment, a person using the table would be apt to look for it. In general, substituted compounds have been listed under the common name of the parent compound. In three cases, large groups of compounds have been brought together under a group heading. These groups are the heme compounds, the indophenols, and the quinones. The naming of all flavins as isoalloxazines gives these systems a common location in the alphabetical list. While such grouping requires some departure from the logic stated above, it has the advantage of showing the user at a glance which of certain closely related compounds are to be found in the table. Many systems are entered under both their trivial name and their systematic name in the alphabetical list.

THE TABLE OF POTENTIALS

Although the values of E'_o at pH 7 are not available for many systems, it was felt that an arrangement in order of increasing potential at this pH was the most desirable. Systems for which E'_o was determined at some other pH are placed in the table according to their estimated potential at pH 7; the values actually quoted are E'_o for the pH nearest 7 for which a value is given by the author quoted.

For a definition of the term E'_o, the reader is referred to the previous chapter.

VARIATION OF POTENTIAL WITH pH

No attempt has been made to present all the available data on the pH variation of the potentials of the redox systems listed in the table. The theoretical considerations in-

*See page 72 for the complete literature references to these authors.

volved in calculating E'_o for various pH values are discussed in the preceding chapter. Workers interested in using indicator dyes at pH's other than 7 should consult "Oxidation-Reduction Potentials in Bacteriology and Biochemistry" by L. F. Hewitt and "Oxidation-Reduction Potentials" by L. Michaelis.

It should be noted that the values of $\frac{\Delta E'_o}{\Delta pH}$ given in the table are for 30° regardless of the temperature at which determinations were actually made. The use of this temperature gives figures which are multiples of 0.03. The values for other temperatures may be obtained by substituting the correct absolute temperature into the factor $\frac{n - a}{n} \frac{RT}{F}$ of equation (5), Chapter III.

LITERATURE CITATIONS

Each reference from which data have been taken is cited only once. When additional listings have been made from the same source, the authors names are given in the reference column, followed by the number, in parentheses, of the system with which the complete citation is given.

ALPHABETICAL LIST OF SYSTEMS

The number which follows each entry in this list is the number assigned to the system in the main table.

Acetaldehyde/acetate 16
Acridine 39
Acridine, 1-amino- 28
Acridine, 2-amino- 18
Acridine, 3-amino- 32
Acridine, 4-amino- 43
Acridine, 5-amino- 9
Acridine, 2,8-diamino- 11
Adrenalin 207
Alanine/ammonium pyruvate 119
Alloxazine 41
Ammonia/hydroxylamine 211
Anthraquinones--see Quinones
Araboflavin 60
Ascorbic acid/dehydroascorbic acid 150
Azure I 134
Bindschedler's green, zinc double salt 182
Brilliant alizarin blue 76
Brilliant cresyl blue chloride 145
Bromide/bromine 219
Butyrate/crotonate 127
Calcium/calcium ion 2
Catechol 206
Catechol, ferro/ferri 198
Chlorate/perchlorate 218
Chloride/chlorine 228
Chlororaphine 92
Chromic ion/dichromate 209
Chromium/chromic ion 12
Chromous ion/chromic ion 25
Ciba scarlet sulfonate 122
Cobalt/cobaltous ion 46
Cobaltous ion/cobaltic ion 5
Coenzyme I 55
Copper/cupric ion 201
Cresyl blue 141
Cresyl violet 77
Cuprous ion/cupric ion 171
Cysteine/Cystine 85
Cytochromes--see under Heme compounds
Dialuric acid/alloxan 115
Dopa 205
Echinochrome A (see also Spinone A) 33
Epinephrine 207
Epinine 203
Ergothioneine 116
Ethanol/acetaldehyde 72
Ethyl Capri blue nitrate 107
Ferrocyanide/ferricyanide 204
Ferrous ion/ferric ion 223
Flavins--see Isoalloxazines
Formaldehyde/formate 15
Formazane, hexyldiphenyl-/tetrazolium chloride, 2,3-diphenyl-5-hexyl- 64
Formate/carbon dioxide 23
Gallocyanine 136
Gallophenin 84
Glucoflavin 59
Glucose/gluconate 18a
Glucose-6-phosphate/6-phosphogluconate 22a
Glutamate/α-ketoglutarate 125
Glutathione 143
Glyceraldehyde-3-phosphate/1,3-diphosphoglycerate 44a
Hallachrome 137

HEME COMPOUNDS

Carbon monoxide hemoglobin/methemeglobin 195

Cyanide hemochromogen 68
Cyanide Spirographis hemochromogen 93
Cytochrome a 197
Cytochrome b 121
Cytochrome c 191
"Cytochrome"/myoglobin 168
Heme, ferro/ferri 63
Spirographis heme 106
Hemocyanin/methemocyanin 216
Hemoglobin/cyanohemoglobin 91
Hemoglobin/methemoglobin 169
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TABLE OF OXIDATION-REDUCTION POTENTIALS

NUMBER	SYSTEM	TEMP.	pH	E'_o (VOLTS)	$\left(\frac{\Delta E'_o}{\Delta pH}\right)_{30°}$	pH RANGE	REFERENCE
1	Potassium/potassium ion	25	0.0	-2.922	0	acid	Latimer and Hildebrand "Reference Book of Inorganic Chemistry," revised ed., Macmillan, 1940, p. 474
2	Calcium/calcium ion	25	0.0	-2.87	0	acid	Latimer and Hildebrand (1)
3	Sodium/sodium ion	25	0.0	-2.712	0	acid	Latimer and Hildebrand (1)
4	Magnesium/magnesium ion	25	0.0	-2.34	0	acid	Latimer and Hildebrand (1)
5	Cobaltous ion/cobaltic ion	25	0.0	-1.84	-	-	Latimer and Hildebrand (1)
6	Water/hydrogen peroxide	25	0.0	-1.77	-	-	Latimer and Hildebrand (1)
7	Manganese/manganous ion	25	0.0	-1.05	0	acid	Latimer and Hildebrand (1)
8	Oxalic acid/carbon dioxide	25	0.0	-0.49	-	-	Latimer and Hildebrand (1)
9	Acridine, 5-amino-	-	7.3	-0.916	-	-	Breyer, Buchanan and Duewell, J. Chem. Soc., 1944, 360
10	Zinc/zinc ion	25	0.0	-0.7620	0	acid	Latimer and Hildebrand (1)
11	Acridine, 2,8-diamino-	-	7.3	-0.731	-	-	Breyer, Buchanan and Duewell (9)
12	Chromium/chromic ion	25	0.0	-0.71	0	acid	Latimer and Hildebrand (1)
13	Pyruvate/acetate-bicarbonate	-	7.0	-0.630 (calc.)[1]	-	-	Kalckar, Chem. Rev. 28, 71 (1941)
14	α-Ketoglutarate/succinate	-	7.0	-0.600 (calc.)[1]	-	-	Kalckar, (13)
15	Formaldehyde/formate	25	14.0	-1.114	-	-	Latimer and Hildebrand (1)
16	Acetaldehyde/acetate	-	7.0	-0.468 (calc.)[1]	-	-	Kalckar (13)
17	Sulfurous acid/sulfate	25	0.0	+0.20	-	-	Latimer and Hildebrand (1)
18	Acridine, 2-amino-	-	7.3	-0.468	-	-	Breyer, Buchanan and Duewell (9)
18a	Glucose/gluconate	-	7.0	-0.45	-	-	Ochoa, in Green, "Currents in Biochemical Research," Interscience, 1946, p. 165
19	Ethyl viologen	-	7.0	-0.449	-	-	Stern, Tab. Biol. Per. 4, 1 (1935)
20	Methyl viologen	-	7.0	-0.446	-	-	Stern (19)
21	Betaine viologen	-	7.0	-0.444	-	-	Stern (19)
22	Iron/ferrous ion	25	0.0	-0.440	-	-	Latimer and Hildebrand (1)
22a	Glucose-6-phosphate/6-phosphogluconate	-	7.0	-0.43	-	-	Barron, Biological Symposia, Lancaster, 10, 27 (1943)
23	Formate/carbon dioxide	30	7.0	-0.420	-	-	Woods, Biochem. J., 30, 515 (1936)
24	Hydrogen/hydrogen ion	all temps.	0.000	0.0000	-.06	entire range	
		30	7.000	-0.4200	-	-	
25	Chromous ion/chromic ion	25	0.0	-0.41	0	acid	Latimer and Hildebrand (1)
26	Hypoxanthine/uric acid	38	7.31	-0.410	-	-	Filitti, J. chim. phys. 32, 1 (1935)
27	Rosindone sulfate	-	7.0	-0.383	-	-	Stern (19)

[1] From thermodynamic considerations.

NUMBER	SYSTEM	TEMP.	pH	E'_o (VOLTS)	$\left(\frac{\Delta E'_o}{\Delta pH}\right)_{30°}$	pH RANGE	REFERENCE
28	Acridine, 1-amino-	-	7.3	-0.394	-	-	Breyer, Buchanan and Duewell (9)
29	Hypoxanthine/xanthine	-	7.0	-0.371	-	-	Green, Biochem. J., 28, 1550 (1934)
30	Xanthine/uric acid	38	7.0	-0.363	-	-	Filitti (26)
31	Benzyl viologen	-	7.0	-0.359	-	-	Stern (19)
32	Acridine, 3-amino-	-	7.3	-0.369	-	-	Breyer, Buchanan and Duewell (9)
33	1,4-Naphthoquinone, 2-ethyl-3,5,6,7,8-pentahydroxy- (Echinochrome A)	-	0.0	+0.080 (Est.[2])	-	-	Wallenfels and Möhle, Ber. 76B, 924 (1943)
34	Neutral violet	-	7.0	-0.340	-	-	Stern (19)
35	Neutral red	-	7.0	-0.340	-	-	Stern (19)
36	Neutral red iodide	-	7.0	-0.330	-	-	Stern (19)
37	Thiophenol	25	7.0	-0.30	-.06	0-7	Ryklan and Schmidt, Univ. Calif. (Berkeley) Pub. Physiol., 8, 257 (1944)
38	Induline scarlet	-	7.0	-0.300	-	-	Stern (19)
38a	Isocitrate/oxalosuccinate	-	7.0	-0.30	-	-	Ochoa (18a)
39	Acridine	-	7.3	-0.313	-	-	Breyer, Buchanan and Duewell (9)
40	L-β-Hydroxybutyrate/acetoacetate	38	7.0	-0.293	-.06	6-8	Hoff-Jørgensen, Skand. Arch. Physiol. (D) 80, 176 (1938)
41	Alloxazine	30	5.0	-0.170	-	-	Michaelis, Schubert and Smythe, J. Biol. Chem., 116, 587, (1936)
42	Safranine T	-	7.0	-0.289	-	-	Stern (19)
43	Acridine, 4-amino-	-	7.3	-0.301	-	-	Breyer, Buchanan and Duewell (9)
44	Rosinduline GG	-	7.0	-0.281	-	-	Stern (19)
44a	Glyceraldehyde-3-phosphate/1, 3-diphosphoglycerate[2a]	-	7.0	-0.28	-	-	Ochoa (18a)
					-.06	5.5-8.3	Meyerhof and Oesper, J. Biol. Chem., 170, 1 (1947)
45	Hydrogen sulfide/sulfur	25	0.0	+0.141	-	-	Latimer and Hildebrand (1)
46	Cobalt/cobaltous ion	25	0.0	-0.277	0	acid	Latimer and Hildebrand (1)
47	Phenosafranine, tetramethyl-	-	7.0	-0.273	-	-	Stern (19)
48	Janus green (Janus red)	-	7.0	-0.256	-	-	Stern (19)
49	Phenosafranine, tetraethyl	-	7.0	-0.254	-	-	Stern (19)
50	Phenosafranine, dimethyl-	-	7.0	-0.252	-	-	Stern (19)
51	Hemoglobin-cyanohemoglobin	-	7.0	-0.252	-	-	Schmidt, Biochem. Z., 296, 210 (1938)
52	Phenosafranine	-	7.0	-0.252	-	-	Stern (19)
53	Isopropyl alcohol/acetone	35	7.0	-0.251	-	-	Wurmser and Filitti, J. chim. phys. 33, 577 (1936)

[2] Estimated on the basis of a study of the effects on the potential of 1,4-naphthoquinone of substituents in various positions in the molecule.

[2a] Potential dependent on the concentration of orthophosphate as well as that of oxidant and reductant.

NUMBER	SYSTEM	TEMP.	pH	E'_o (VOLTS)	$\left(\frac{\Delta E'_o}{\Delta pH}\right)_{30°}$	pH RANGE	REFERENCE
54	Anthraquinone-2-sulfonic acid	-	7.0	-0.250	-	-	Stern (19)
55	Coenzyme I	30	7.0	-0.282	-.03	-	Borsook, J. Biol. Chem. 133, 629 (1940)
56	Spinone A	-	0.0	+0.175	-	-	Wallenfels and Möhle (33)
57	Isoalloxazine, 6,7,9-trimethyl (Lumiflavin)	20	7.0	-0.207	-.06	0.4-6.0	Kuhn and Boulanger, Ber. 69B, 1557 (1936)
					-.03	7.0-9.0	
					-.06	10.0-12.8	
		30	7.0	-0.223	-	-	Michaelis, Schubert and Smythe (41)
58	Isoalloxazine, 6,7-dimethyl-9-D-ribityl-(Riboflavin)	20	7.0	-0.186	-.06	.4-6.5	Kuhn and Boulanger (57)
					-.03	6.5-9.0	
					-.06	10.0-12.8	
		30	7.0	-0.208	-	-	Michaelis, Schubert and Smythe (41)
59	Isoalloxazine, 6,7-dimethyl-9-D-glucityl-(Glucoflavin)	30	7.0	-0.208	-	-	Michaelis, Schubert and Smythe (41)
60	Isolloxazine, 6,7-dimethyl-9-D-arabityl-(araboflavin)	30	7.0	-0.208	-	-	Michaelis, Schubert and Smythe (41)
61	Vanadous ion/vanadic ion	25	0.0	-0.20	-	-	Latimer and Hildebrand (1)
62	Anthraquinone-1,5-disulfonic acid	-	7.0	-0.200	-	-	Stern (19)
63	Heme, ferro/ferri	30	8.18 ($B_4O_7^{=}$)	-0.188	-	-	Barron, J. Biol. Chem. 121, 285 (1937) ibid. 133, 51 (1940)
			8.18 ($PO_4^{=}$)	-0.226			
					-.06	7.0-9.2	Barron, Science, 85, 48 (1938)
					-.09	9.2-12.0	
64	Formazane, hexyldiphenyl/tetrazolium chloride, 2,3-diphenyl-5-hexyl-	20	7.0	-0.20 (ARP[3])	-	-	Kuhn and Jerchel, Ber. 74B, 949 (1941)
65	Neutral blue	-	7.0	-0.192	-	-	Stern (19)
66	Isoalloxazine, 6,7-dimethyl-9-D-ribityl-5'-phosphate (Riboflavin phosphate)	20	7.0	-0.185	-	-	Kuhn and Boulanger (57)
67	1,4-Naphthoquinone, 2-hydroxy-3-(3-methyl-4-hydroxybut-2-enyl)-(Lomatiol)	30	7.1	-0.191	-	-	Hill, J. Am. Chem. Soc. 60, 1990 (1938)
68	Cyanide hemochromogen	30	9.89	-0.183	0	-	Barron and Hastings, J. Biol. Chem. 109, iv (1935)
69	1,4-Naphthoquinone, 2-hydroxy-3-(3-methylbut-2-enyl)-(Lapachol)	-	7.0	-0.180	-	-	Hill (67)
70	Lactate/pyruvate	35	7.01	-0.180	-	-	Barron and Hastings, J. Biol. Chem. 107, 567 (1934)

[3] ARP, apparent reduction potential. The system does not form a reversible couple as far as is known. The value given is based on experiments with dyes.

NUMBER	SYSTEM	TEMP.	pH	E'_o (VOLTS)	$\left(\frac{\Delta E'_o}{\Delta pH}\right)_{30°}$	pH RANGE	REFERENCE
71	Isoalloxazine, 9-methyl-	20	7.0	-0.167	-	0.40-12.8	Kuhn and Boulanger (57)
		30	7.0	-0.183	-	-	Michaelis, Schubert and Smythe (41)
72	Ethanol/acetaldehyde	25	7.0	-0.200	-	-	Wurmser and Filitti-Wurmser (53)
		25	7.0	-0.163 (calc[1])	-	-	Kalckar (13)
73	1,4-Naphthoquinone, 2-methyl-3-hydroxy- (Phthiocol)	-	7.0	-0.180	-	-	Hill, Proc. Soc. Exptl. Biol. Med. 35, 363 (1936)
74	Isoalloxazine, 6,9-dimethyl-	20	7.0	-0.176	-	0.4-12.8	Kuhn and Boulanger (57)
75	Isoalloxazine, 3,9-dimethyl-	20	7.0	-0.175	-	0.4-12.8	Kuhn and Boulanger (57)
76	Brilliant alizarin blue	-	7.0	-0.173	-	-	Stern (19)
77	Cresyl violet	-	7.0	-0.166	-	-	Stern (19)
78	Isoalloxazine, 9-D-glucityl-	30	7.0	-0.163	-	-	Michaelis, Schubert and Smythe (41)
79	Isoalloxazine, 9-hydroxyethyl-	20	7.0	-0.156	-	0.4-12.8	Kuhn and Boulanger (57)
80	Indigosulfonic acid	-	7.0	-0.150	-	-	Stern (19)
81	Isoalloxazine, 3,6,7-trimethyl-9-D-glucityl-	20	4.70	-0.080	-	-	Kuhn and Boulanger (57)
82	Isoalloxazine, 6,7-dimethyl-9-D-xylityl- (xyloflavin)	20	4.70	-0.078	-	-	Kuhn and Boulanger (57)
83	1,4-Naphthoquinone, 2-hydroxy-(Lawsone)	-	7.18	-0.154	-.06 -.09	0.36-3.50 4.0-9.18	Friedheim, Biochem. J. 28, 180 (1934)
		25	0.0	+0.357 (50% EtOH)	-	-	International Critical Tables, vol. 6, p. 34
84	Gallophenin	-	7.0	-0.142	-	-	Stern (19)
85	Cysteine/cystine	25	7.0	-0.14	-.06	0-7	Ryklan and Schmidt (37)
86	Thioglycollic acid	25	7.0	-0.14	-.06	0-7	Ryklan and Schmidt (37)
87	Isoalloxazine, 9-dihydroxypropyl-	20	7.0	-0.134	-.06 -.03 -.06	0.4-6.0 6.0-8.0 9.0-12.8	Kuhn and Boulanger (57)
88	Isoalloxazine, 9-phenyl-	20 20	7.0 4.70	-0.126 0.000	-	0.4-12.8	Kuhn and Boulanger (57)
89	Indigodisulfonic acid (Indigo carmine)	-	7.0	-0.125	-	-	Stern (19)
90	Nile blue	-	7.0	-0.116 to -0.150	-	-	Stern (19)
91	Valine/ammonium dimethylpyruvate[4]	37	7.0	-0.115	-	-	Wurmser and Filitti-Wurmser, Enzymologia 7, 161 (1939)
92	Chlororaphine	29.5	7.42	-0.139	-.06	2-10	Elema, Rec. Trav. Chim. 52, 569 (1933)
93	Cyanide Spirographis hemochromogen	30	9.95	-0.113	-	-	Barron, J. Biol. Chem. 133, 51 (1940)
94	o-Thiocresol	25	7.0	-0.11	-.06	0-7	Ryklan and Schmidt (37)
95	Isoalloxazine, 6,8,9-trimethyl-	20	7.0	-0.109	-	0.4-12.8	Kuhn and Boulanger (57)

[4] Potential dependent on the activity of ammonium ion as well as that of oxidant and reductant.

NUMBER	SYSTEM	TEMP.	pH	E'_o (VOLTS)	$\left(\frac{\Delta E'_o}{\Delta pH}\right)_{30°}$	pH RANGE	REFERENCE
96	Isoalloxazine, 9-benzyl-	20	4.70	-0.036	-	-	Kuhn and Boulanger (57)
97	Isoalloxazine-9-acetic acid	20	4.70	-0.036	-	-	Kuhn and Boulanger (57)
98	Pigment of Chromodoris zebra	30	7.0	-0.102	-	-	Preisler, J. Gen. Physiol. 13, 349 (1930)
99	Malate/oxalacetate	38	7.0	-0.102	-.06	7.47-9.15	Lehmann and Jorgensen, Skand. Arch. Physiol. 82, 113 (1939)
100	Succinate/maleate	37	7.0	-0.094	-	-	Laki, Z. physiol. Chem. 236, 31 (1935)
101	Thiolhistidine	25	7.0	-0.09	-.06	0-7	Ryklan and Schmidt (37)
102	Hydrocyanic acid/ cyanogen	25	0.0	+0.33	-	-	Latimer and Hildebrand (1)
103	Oxytocic hormone	18	6.0	-0.025	-	-	Gulland and Randall, Biochem. J. 29, 378 (1935)
104	Isoalloxazine, 9-cyclohexyl-	20	4.70	-0.012	-	-	Kuhn and Boulanger (57)
105	Indigotrisulfonic acid	-	7.0	-0.081	-	-	Stern (19)
106	Spirographis heme	30	7.22	-0.089	-.06	7.2-9.6	Barron (93)
107	Ethyl Capri blue nitrate	-	7.0	-0.072	-	-	Stern (19)
108	α-Hydroxyglutarate/ α-ketoglutarate	-	7.0	-0.07 (approx.)	-	-	Weil-Malherbe, Biochem. J. 31, 2080 (1937)
109	Sulfur/sulfurous acid	25	0.0	+0.45	-	-	Latimer and Hildebrand (1)
110	1,4-Naphthoquinone, 2-hydroxy-3-chloro-	25	0.0	+0.352 (50% EtOH)	-	-	International Critical Tables (83)
111	Yellow enzyme	38	7.0	-0.060	-	-	Kuhn and Boulanger (57)
112	Thio ethylene glycol (mono)	25	7.0	-0.06	-.06	0-7	Ryklan and Schmidt (37)
113	Methyl Capri blue	-	7.0	-0.061	-	-	Stern (19)
114	1,4-Naphthoquinone, 2-methyl-3-phytyl- (Vitamin K_1)	20	0.0	+0.363 (95% EtOH)	-	-	Riegel, Smith and Schweitzer, J. Am. Chem. Soc. 62, 992 (1940)
115	Dialuric acid/alloxan	25	0.0	+0.3672	-	-	Biilman and Berg, Ber. 63B, 2188 (1930)
116	Ergothioneine	25	7.0	-0.05	-.06	0-7	Ryklan and Schmidt (37)
117	Luciferin (Photobacterium phosphoreum)	18	7.0	-0.050 (Est.[5])	-	-	Spruit and Schuiling, Rec. Trav. Chim. 64, 219 (1945)
118	Toxoflavin (Bact. boukrek)	30	7.0	-0.049	-	-	Stern, Biochem. J. 29, 500 (1935)
119	Alanine/ammonium pyruvate[4]	37	7.0	-0.048	-	-	Wurmser and Filitti-Wurmser, Compt. rend. Soc. Biol. 129, 133 (1938)
120	Indigotetrasulfonic acid	-	7.0	-0.046	-	-	Stern (19)
121	Cytochrome b	20	7.4	-0.04	-	-	Ball, Biochem. Z. 295, 262 (1938)
122	Ciba scarlet sulfonate	-	7.0	-0.036	-	-	Hewitt, "Oxidation-Reduction Potentials in Bacteriology and Biochemistry," 3rd ed., London County Council, 1935
123	Iron/ferric ion	25	0.0	-0.036	0	acid	Latimer and Hildebrand (1)
124	Pyocyanine	30	7.0	-0.034	-.06 -.03	5-9 10-11.5	Friedheim and Michaelis, J. Biol. Chem. 91, 355 (1931)

[5] Estimated on the basis of a study of the ability of redox active compounds to inhibit light production by the bacterium.

NUMBER	SYSTEM	TEMP.	pH	E'_o (VOLTS)	$\left(\frac{\Delta E'_o}{\Delta pH}\right)_{30°}$	pH RANGE	REFERENCE
125	Glutamate/α-keto-glutarate	-	7.0	-0.030 (calc.)[1]	-	-	Kalckar (13)
126	Hermidin/cyanohermidin	30	7.3	-0.045	-	-	Cannan, Biochem. J. 20, 927 (1926)
127	Butyrate/crotonate	-	7.0	-0.025	-	-	Kalckar (13)
128	Pilocarpine hemochromogen	30	9.63	-0.170	-.06	-	Barron (93)
129	Succinate/fumarate	-	7.0	0.00	-	-	Kalckar (13)
130	1,4-Naphthoquinone, 2-methyl-	-	0.0	0.422	-	-	Wallenfels and Möhle (33)
131	Propionate/acrylate	-	7.0	0.010 (calc.[1])	-	-	Kalckar (13)
132	Toluidine blue	-	7.0	0.011	-	-	Stern (19)
133	Methylene blue	-	7.0	0.011	-.09 -.06	<5 >6	Stern (19)
134	Azure I	-	7.0	0.011	-	-	Stern (19)
135	Histidine hemochromogen	-	9.5	-0.138	-.06	-	Barron, Science 85, 58 (1938)
136	Gallocyanine	-	7.0	0.021	-	-	Stern (19)
137	Hallachrome	20	7.0	0.022	-	-	Friedheim (83)
138	Pigment of Arion rufus	20	7.0	0.025	-	-	Friedheim (83)
139	Palmitate/oleate	-	7.0	0.025	-	-	Kalckar (13)
140	Indole-2-carboxylic acid, 2,3-dihydro-4,6-dihydroxy-	17	4.6	0.171	-	-	Friedheim, Naturwissenschaften 21, 177 (1933)
141	Cresyl blue	-	7.0	0.031	-	-	Rapkine, Struyck and Wurmser, J. chim. phys. 26, 340 (1929)
		-	7.0	0.047	-	-	Cohen and Preisler, Suppl. 92, Pub. Health Rep. 1931
142	1,4-Naphthoquinone	-	0.0	0.488	-	-	Wallenfels and Möhle (33)
		-	7.0	0.036	-	-	Stern (19)
143	Glutathione	25	7.0	0.04	-.06	0-7	Ryklan and Schmidt (37)
144	1,4-Naphthoquinone, 5-hydroxy- (Juglone)	-	7.32	0.023	-.06 -.03 -.06	2.0-6.2 6.7-7.5 8.0-10.0	Friedheim (83)
		-	0.0	0.450	-	-	Wallenfels and Möhle (33)
145	Brilliant cresyl blue chloride	-	7.0	0.045	-	-	Stern (19)
146	Myoglobin/metmyoglobin	30	7.0	0.046	-.07	5.9-7.4	Taylor and Morgan, J. Biol. Chem. 144, 15 (1942)
147	Phoenicin	20	7.0	0.047	-	-	Friedheim (83)
148	Resazurin	-	7.0	0.051	-	-	Twigg, Nature 155, 401 (1945)
149	Thionine (Lauth's Violet)	-	7.0	0.062	-	-	Stern (19)
150	Ascorbic acid/dehydroascorbic acid	35.5	3.30	0.204	-.06	2.04-4.2	Borsook et al., J. Biol. Chem. 117, 237 (1937)
			6.43	0.080 (Extrap.[6])	-.03	4.2-6.4	
151	1,4-Naphthoquinone, 2-chloro-	-	0.0	0.508	-	-	Wallenfels and Möhle (33)

[6] Calculated from the determined values by correcting for the pH dependent irreversible hydrolysis of dehydroascorbic acid above pH 4.2.

NUMBER	SYSTEM	TEMP	pH	E'_o (VOLTS)	$\left(\frac{\Delta E'_o}{\Delta pH}\right)_{30°}$	pH RANGE	REFERENCE
152	Pilocarpin Spirographis hemochromogen	30	9.63	-0.067	-	-	Barron (93)
153	2-Naphthol, 1-hydroxyl-amino-/1-nitroso-	25	0.0	0.52	-	-	International Critical Tables (83)
154	Hemoglobin/oxyhemoglobin	-	7.0	0.102	-	-	Schmidt (51)
155	1,4-Naphthoquinone-2-sulfonic acid	24	0.0	0.534	-	-	International Critical Tables (83)
156	Toluylene blue chloride	-	7.0	0.115	-	-	Stern (19)
157	2-Sulfo-1-naphtholindo-3',5'-dichlorophenol	-	7.0	0.119	-	-	Stern (19)
158	2-Sulfo-1-naphtholindo-2',6'-dichlorophenol	-	7.0	0.119	-	-	Stern (19)
159	Reductinic acid	23	6.81	0.122	-.03	5-7	Mayer, J. chim. phys. 34, 109 (1937)
160	2-Sulfo-1-naphtholindo-phenol	-	7.0	0.123	-	-	Stern (19)
161	m-Toluenediamine-indophenol chloride	-	7.0	0.127	-	-	Stern (19)
162	2-Sulfo-1-naphtholindo-phenol-3'-sulfonic acid	-	7.0	0.135	-	-	Stern (19)
163	α-Picoline hemochromogen	-	9.63	-0.033	-.06	-	Barron (93)
164	1,2 Naphthoquinone	-	7.0	0.143 (Extrap.7)	-	-	Stern (19)
		-	0.0	0.578	-	-	Wallenfels and Mohle (33)
165	α-Picoline Spirographis hemochromogen	30	9.63	-0.010	-	-	Barron (93)
166	Isoalloxazine, 5,6-benzo-9-methyl-	20	12.8	-0.50	-	-	Kuhn and Boulanger (57)
167	Guaiacolindo-2,6-dibromo-phenol	-	7.0	0.159	-	-	Stern (19)
168	"Cytochrome"/myoglobin	-	7.4	0.16	-	-	Bechtold and Pfeilsticker, Biochem. Z. 307, 194 (1941)
169	Hemoglobin/methemoglobin[8]	10	5.0-6.6	0.200	-	-	Havermann, Biochem. Z. 314, 118 (1943)
		45	5.0-5.6	0.140	-	-	
170	Pyridine hemochromogen	-	9.5	0.017	-.06	-	Barron (135)
171	Cuprous ion/cupric ion	25	0.0	0.167	0	acid	Latimer and Hildebrand (1)
172	Thymolindophenol	-	7.0	0.174	-	-	Hewitt (122)
173	Phenylhydroxylamine/nitrosobenzene	25	0.0	0.60	-	-	International Critical Tables (83)
174	2,6-Dichlorophenolindo-o-cresol	-	7.0	0.181	-	-	Stern (19)
175	o-Cresolindophenol	-	7.0	0.195	-	-	Stern (19)
176	Murexide	20	4.7	0.151	-.06	-	Kuhn and Lyman, Ber. 69, 1547 (1936)
177	Nicotine hemochromogen	-	9.5	0.053	-.06	-	Barron (135)
178	m-Cresolindophenol	-	7.0	0.210	-	-	Stern (19)
179	2,6-Dichlorophenolindo-phenol	-	7.0	0.217	-	-	Stern (19)
180	2,6-Dibromophenolindo-phenol	-	7.0	0.217	-	-	Stern (19)

[7] Extrapolated. The compound is unstable above pH 6.2.

[8] The potential was found to have an unusual dependence on temperature. Values were not constant over the pH range studied, but showed no consistent tendency to decrease or increase.

NUMBER	SYSTEM	TEMP.	pH	E'_o (VOLTS)	$\left(\frac{\Delta E'_o}{\Delta pH}\right)_{30°}$	pH RANGE	REFERENCE
181	2,6-Dichlorophenolindo-o-chlorophenol	-	7.0	0.218	-	-	Hewitt (122)
182	Bindschedler's green, zinc double salt	-	7.0	0.224	-	-	Stern (19)
183	Phenol blue chloride	-	7.0	0.227	-	-	Stern (19)
184	o-Bromophenolindophenol	-	7.0	0.231	-	-	Stern (19)
185	o-Chlorophenolindophenol	-	7.0	0.233	-	-	Stern (19)
186	2-Sulfophenolindo-2',6'-dibromophenol	-	7.0	0.235	-	-	Stern (19)
187	m-Bromophenolindophenol	-	7.0	0.248	-	-	Stern (19)
188	3-Sulfophenolindophenol	-	7.0	0.250	-	-	Stern (19)
189	m-Chlorophenolindo-2,6-dichlorophenol	-	7.0	0.254	-	-	Hewitt (122)
190	Homogentisic acid	-	6.994	0.261	-	-	Fishberg and Dolin, J. Biol. Chem. 101, 159 (1933)
191	Cytochrome c	-	7.0	0.262	0	5.0-8.0	Stotz, Sidwell and Hogness, J. Biol. Chem. 124, 11 (1938)
192	Hydrogen peroxide/oxygen	25	0.0	0.682	-	-	Latimer and Hildebrand (1)
193	3-Sulfophenolindo-2',6'-dibromophenol	-	7.0	0.273	-	-	Stern (19)
194	Quinone (p-Quinone)	25	0.0	0.6994	-.06	<8	Getman and Daniels, "Outlines of Physical Chemistry," 7th ed., Wiley, 1943, p. 438
195	Carbon monoxide hemoglobin/methemoglobin	-	7.0	0.280	-	-	Schmidt (51)
196	Quinone, 2-chloro-	25	0.0	0.7125	-	-	International Critical Tables (83)
197	Cytochrome a	20	7.4	0.29	-	-	Ball (121)
198	Catechol, ferro-/ferri-	20	5.0	0.293	-	-	Laki and Papp, Z. physiol. Chem. 254, 175 (1938)
199	Quinone, 2,6-dichloro	25	0.0	0.722	-	-	International Critical Tables (83)
200	Vanadic ion/vanadyl ion	25	0.0	0.314	-	-	Latimer and Hildebrand (1)
201	Copper/cupric ion	25	0.0	0.3448	0	acid	Latimer and Hildebrand (1)
202	Thiocyanic acid/thiocyanogen	25	0.0	0.77	-	-	Latimer and Hildebrand (1)
203	Epinine	30	7.664	0.306	-	-	Ball and Chen. J. Biol. Chem. 102, 691 (1933)
204	Ferrocyanide/ferricyanide	25	0.0	0.36	-	-	Latimer and Hildebrand (1)
205	Phenylalanine, L-3,4-dihydroxy-(Dopa)	30	7.0	0.370	-	-	Ball and Chen (203)
206	o-Quinone	30	7.66	0.333	-	-	Ball and Chen (203)
		-	0.0	0.794	-.06	-	Fieser and Peters, J. Am. Chem. Soc. 53, 793 (1931)
207	Adrenalin	30	7.0	0.380	-.06	-	Ball and Chen (203)
208	Hemoglobin/nitric oxide hemoglobin	-	7.0	0.380 (approx.)	-	-	Schmidt (51)
209	Chromic ion/dichromate	25	0.0	1.36	-	-	Latimer and Hildebrand (1)
210	Nitrosoguanidine/nitroguanidine	-	0.0	0.85	-	-	Hahn et al., J. Am. Chem. Soc. 66, 1223 (1944)
211	Ammonia/hydroxylamine	25	14.0	0.42	-	-	Latimer and Hildebrand (1)
		25	0.0	1.35	-	-	
212	Sulfide/sulfur	25	14.0	0.508	-	-	Latimer and Hildebrand (1)

NUMBER	SYSTEM	TEMP.	pH	E'_o (VOLTS)	$\left(\frac{\Delta E'_o}{\Delta pH}\right)_{30°}$	pH RANGE	REFERENCE
213	Porphyrindine, spiro-cyclohexyl analog	18	7.0	0.534	-	-	Porter and Hellerman,,J. Am. Chem. Soc. 66, 1653 (1944)
214	Iodide/iodine	25	0.0	0.5345	0	acid	Latimer and Hildebrand (1)
215	Iodide/triiodide	25	0.0	0.5355	0	acid	Latimer and Hildebrand (1)
216	Hemocyanin/methemo-cyanin	-	7.0	0.540	-	-	Conant, Chow and Schonbach J. Biol. Chem. 101, 463 (1933)
217	Porphyrindine	18	7.0	0.565	-	-	Kuhn and Franke, Ber. 68, 1528 (1935)
218	Chlorate/perchlorate	25	0.0	1.00	-	-	Latimer and Hildebrand (1)
219	Bromide/bromine	25	0.0	0.652	0	acid	Latimer and Hildebrand (1)
220	Porphyrexide, spiro-cyclohexyl analog	18	7.0	0.690	-.06	1.93-5.0	Porter and Hellerman (213)
221	Porphyrexide	18	7.0	0.725	-	-	Kuhn and Franke (217)
222	Selenium/selenious acid	25	0.0	0.740	-	-	Latimer and Hildebrand (1)
223	Ferrous ion/ferric ion	25	0.0	0.771	0	acid	Latimer and Hildebrand (1)
224	Water/oxygen	25	7.0	0.815	-.06	-	Latimer and Hildebrand (1)
225	Nitrous acid/nitrate	25	0.0	0.96	-	-	Latimer and Hildebrand (1)
226	Vanadyl ion/vanadate	25	0.0	1.000	-	-	Latimer and Hildebrand (1)
227	Manganous ion/manganese dioxide	25	0.0	1.28	-	-	Latimer and Hildebrand (1)
228	Chloride/chlorine	25	0.0	1.3583	0	acid	Latimer and Hildebrand (1)
229	Manganous ion/manganic ion	25	0.0	1.51	-	-	Latimer and Hildebrand (1)
230	Manganous ion/per-manganate	25	0.0	1.52	-	-	Latimer and Hildebrand (1)
231	Oxygen/ozone	25 25	14.0 0.0	1.4 2.07	- -	- -	Latimer and Hildebrand (1)

Chapter V

COENZYMES AND ACTIVATORS

by

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INTRODUCTION

In addition to substrate and enzyme, many enzymatic reactions require another type of agent, referred to as a "coenzyme". The term was first used by Bertrand in 1897, to refer to certain inorganic ions which activated enzymes; for example the activation of pancreatic diastase by chlorides and of rennin by calcium. However, the term coenzymes will be restricted here to the more modern usage, in which they are defined as heat-stable, dialyzable organic substances required for the activity of their respective enzymes. The first observation of these was made by Harden and Young in 1906, when they found that the diffusible fraction of boiled yeast juice greatly stimulated fermentation in cell-free extracts. This yeast fermentation coenzyme, then termed "cozymase", was found in many tissues other than yeast including animal organs and milk (Meyerhof, 1918). This factor has since been found to function with specific enzyme systems, as for example, the lactic dehydrogenase (Szent-Gyorgyi, 1925; Banga et al, 1932-1933).

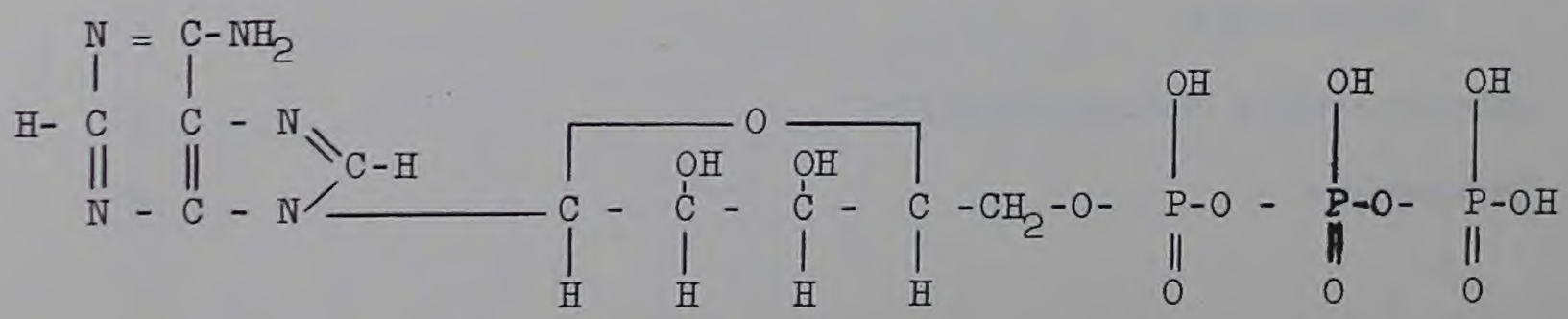
At this time it is possible to describe the function of many of the vitamins in terms of their places in the structures of coenzymes, and new functions are still being found for some of the well known coenzymes beyond the functions first observed for them.

ADENOSINE PHOSPHATES

These answer the definition of coenzymes in that they are necessary cofactors in the transfer of phosphate in the cell and in particular in the transfer of high energy phosphate bonds. The system, which to the author's knowledge has been found in every tissue critically examined for it, consists of the three components, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenylic acid (AMP). This phosphate transfer system has been studied by Lohmann, Meyerhof, Embden, Parnas, Needham, Lipmann, Cori, Kalckar, Potter and others. As a result it is known to be essential to the transfer of phosphate bond energy in a wide variety of reactions in many different types of cells.

The best source of these compounds for preparative purposes is skeletal muscle. In resting muscle ATP is found to be 200-300 mg. per cent. ATP is the form most readily isolated and purified. A procedure for this was described by Lohmann in 1928. Since then modifications of the procedure have been described by Kerr (1941a), Needham (1942) and LePage (1945). The yields and ease of isolation of ATP make it best to prepare the homologues from it rather than attempt their separate isolation. Lohmann and Schuster (1935) described a procedure for preparing ADP from ATP by enzymatic hydrolysis with a washed lobster muscle preparation. Conditions for this are described by LePage (1945). Kerr (1941b) has described a method for preparation of AMP from ATP by hydrolysis with barium hydroxide.

FORMULA I



ATP M.W. = 507.

ADP (1 less phosphate group) M.W. = 427.

AMP (2 less phosphate groups) M.W. = 347.

These are relatively labile compounds in solution, quite stable when dry. Neutral solutions are stable in the cold for at least several days. In dilute alkali at 100° C., the pyrophosphate bonds of ATP and ADP are split, AMP is stable. In 1 N HCl at 100° C. the pyrophosphate bonds of ATP and ADP are all broken within 7 min.; and within 10 min. the adenine-ribose bond is also completely destroyed. The resulting ribose phosphate is much more slowly hydrolyzed.

Determination of these compounds in tissues necessarily involves use of in situ freezing, to prevent rapid changes caused by stimulation,-and extraction with a protein precipitant such as trichloroacetic acid or perchloric acid. Fixing of in vitro enzymatic reaction mixtures merely requires addition of the acid to destroy enzyme activity. The acid extracts are neutralized and fractionated with barium and alcohol. In the presence of inorganic phosphorus the ATP and ADP are in the barium precipitates made at slightly alkaline pH. They can be estimated and differentiated by measurement of phosphorus hydrolyzed by 1 N HCl in 7 min. at 100° C. (ATP-P 67 per cent, ADP-P 50 per cent hydrolyzed) and ribose. AMP is found in the fraction precipitated by barium and alcohol and can be estimated by measurement of absorption at 260 mμ, where it exhibits an absorption maximum.

Examples of the function of these "co-phosphorylases" are:

$$\text{ATP + glucose} \longrightarrow \text{glucose-6-phos. + ADP}$$

$$\text{Phosphopyruvic acid + ADP} \rightleftharpoons \text{ATP + pyruvic acid}$$

$$\text{Phosphopyruvic acid + AMP} \rightleftharpoons \text{ADP + pyruvic acid}$$

VITAMIN COMPLEXES

NICOTINIC ACID

This compound is well known as a vitamin, and has been found to function by reason of its place in the structure of two important coenzymes, diphosphopyridine nucleotide (DPN, Co I, cozymase) and triphosphopyridine nucleotide (TPN, Co II). These coenzymes appear to function in the dehydrogenase activity of a wide variety of tissues. They are both found in varying amounts in all the actively metabolizing tissues of higher forms of life and in a variety of microorganisms (Myrback, 1933, 1937; Warburg, 1936). Yeast and liver are the best sources for DPN and TPN preparation respectively. As mentioned earlier, DPN was first discovered in yeast. TPN was first isolated from red blood cells by Warburg et al (1933a, b).

The pyridine nucleotides are very soluble in water, have very limited solubility in organic solvents such as acetone and alcohol, are insoluble in ethyl acetate and fat solvents generally. They readily diffuse through membranes. The molecular weights of DPN and TPN are 663 and 743 respectively. They both form insoluble salts with heavy metals such as Pb,Hg,Ag, but both have water soluble barium salts. They can be separated by use of difference in the solubility of their barium salts in water-alcohol. When three volumes of alcohol are added to a water solution of the two coenzymes at pH9 in the cold, DPN is soluble to the extent of 5 mg. per ml. or greater, while TPN is quantitatively precipitated.

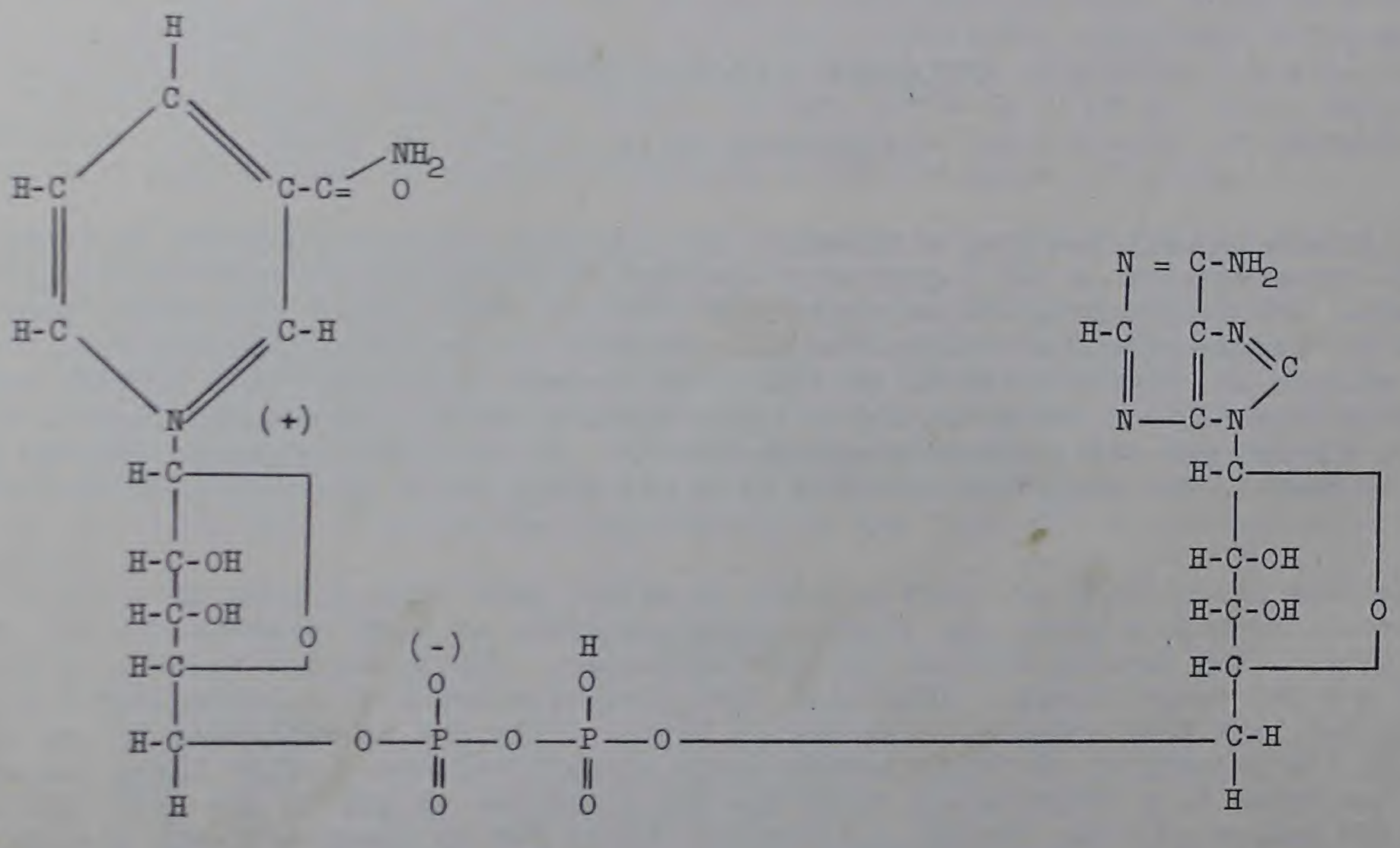
Aqueous solutions of the pyridine nycleotides are acid, DPN having only one free, titratable acid group, TPN having three. The oxidized forms are stable in acid, labile in alkali. The reduced forms show just the reverse, are labile to acid, relatively stable to alkali. Data provided by Schlenk (1941) illustrate this and are given in Table 1.

TABLE 1

Stability of Pyridine Nycleotides

Conditions	DPN		TPN	
	Oxidized	Reduced	Oxidized	Reduced
0.1 N HCl	50% destroyed in 8 min. at 100° C.	Destroyed immediately at 20° C.	50% destroyed in 7.3 min. at 100° C.	Destroyed immediately at 20° C.
0.1 N NaOH	50% destroyed in 17 min. at 20° C.	Slight decrease in activity after 10 min. at 100° C. Stable at 20° C.	50% destroyed after 12 min. at 23° C.	Stable at 20° C.

FORMULA II



Diphosphopyridine Nucleotide

The chemical constituents of both these coenzymes have been established by Warburg and Euler and their co-workers. The two differ only in that TPN possesses one more phosphoric acid group. The structure of DPN has now been well established, but there is less information concerning TPN. The position of the third phosphoric acid group in the latter is uncertain. Schlenk and Euler (1936) proposed a structure for DPN which has been established as correct. Schlenk (1942) provided the last necessary data when he established that d-ribose was the sugar in both positions in the DPN molecule, and that the linkages were ribose-5-phosphate linkages. The structure of DPN is given below. The constituents, adenine, 2 riboses, nicotinamide and 2 phosphoric acids are linked by removal of five mole-

cules of water. It has been demonstrated by Adler et al (1940) that DPN can be converted enzymatically to TPN. The enzymatic synthesis of TPN from nicotinamide, ribose and ATP by pigeon liver extracts has recently been demonstrated by Altman and Evans (1947).

These coenzymes can be adsorbed on alumina and eluted with phosphoric acid. This is a method of separating the two since TPN is held more firmly than DPN (Euler and Schlenk, 1936).

The most reactive part of the molecule, both chemically and biologically, is the nicotinamide residue, which readily and reversibly takes on hydrogen (electrons). Both nucleotides are easily reduced by sodium hydrosulfite, forming dihydrides. In alkaline solution a transient yellow color appears during reduction. This was suggested by Adler et al (1936) to be an intermediate stage where one electron has been accepted and a "free radical" exists. The reduced coenzymes possess an absorption maximum at 340 mμ which is absent from the spectrum of the oxidized forms. Both DPN and TPN are rapidly destroyed by ultraviolet light.

Several methods are available for determination of DPN. Mȳrback (1928) found that yeast fermentation could be used as a measure of DPN when the DPN of the yeast preparation was washed away with cold water. Jandorf et al (1941) provided another biological method in which glycolysis was measured with DPN the limiting factor; the reaction involved was the conversion of hexosediphosphate to phosphoglyceric acid and glycerophosphate. Chemical methods, equally applicable to both pyridine coenzymes, have been used, in which the coenzyme was reduced with hydrosulfite and absorption at 340 mμ measured. One such procedure was recently described by LePage (1947). This does not differentiate between the two nucleotides, but is satisfactory where only one is present.

In this assay, 0.10 ml. of a 1 per cent solution of the material to be assayed is added to each of two 150 x 15 mm. test tubes. To the first is added 0.40 ml. of water and 0.50 ml. of a freshly prepared 1 per cent sodium bicarbonate solution. To the second is added 0.20 ml. of water, 0.50 ml. of the bicarbonate solution and 0.20 ml. of a freshly prepared solution containing 3 per cent sodium hydrosulfite in 1 per cent sodium bicarbonate. The solutions are incubated 20 minutes at room temperature; then to each is added 9.0 ml. of a 1 per cent sodium bicarbonate-1 per cent sodium carbonate solution. They are aerated vigorously for 5 minutes, then absorption is measured at 340 mμ and with a 1 cm. cell. The extinction coefficient X 118 = %DPN*.

TPN can be measured biologically with a system consisting of glucose-6-phosphate and its dehydrogenase, yellow enzyme and oxygen. The rate of oxygen uptake is proportional to the TPN added. This is the method of Warburg et al (1935a).

A number of procedures are available for preparation of DPN. Comments on these were recently made by Schlenk and Schlenk (1947). The most recent method, by LePage (1947), like most of the others uses yeast as a starting material. It provides a relatively simple procedure which gives high yields of DPN, roughly 67 per cent pure. The impurity, which is adenylic acid, is relatively easily removed if desired. The essential steps are as follows: fresh baker's yeast is crumbled into water held at 90 - 92° C. The suspension is cooled and filtered. The filtrate is treated with basic lead acetate, the precipitate filtered off and discarded. DPN is precipitated from this filtrate with silver. The silver salt is decomposed with hydrogen sulfide and DPN precipitated as the free acid by addition of acetone. This crude preparation is purified by fractionatron with lead in alcohol-water solution, precipitated as free acid with acetone, then adsorbed on charcoal and eluted with pyridine - water. Precipitation of this material now gives the 67 per cent purity previously mentioned. The final purification for removal of adenylic acid is accomplished by solution of the DPN in acid methanol and precipitation with ethyl acetate.

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*This includes use of the latest molecular extinction coefficient available to us in the literature (1938), but by personal communication from Prof. O. Meyerhof, we are informed that P. Ohlmeyer has obtained DPN of purity such that the molecular extinction coefficient should be raised by some 11 per cent.

Yields as high as 8 gms. per 100 lbs. of pressed baker's yeast have been obtained with assay over 90 per cent. No loss of DPN occurs in this final stage if it is carried out in the cold.

TPN preparation has not been as extensively studied, and yields of this coenzyme are low. One method available in the literature is that of Warburg et al (1935c), in which horse or beef liver is used as source material. The liver tissue is ground and added to water at 80° C. The suspension is filtered and the filtrate treated with trichloroacetic acid. The protein-free filtrate is neutralized and precipitated with mercuric acetate. The mercury precipitate is decomposed with hydrogen sulfide and the acids precipitated with acetone. Fractionation with barium and alcohol permits removal of DPN and the bulk of other impurities. Then the TPN is dissolved in acid methanol and precipitated with ethyl acetate. Further purification is achieved by fractionation with lead in alcohol-water mixtures and by phosphotungstic acid precipitation.

DPN functions as a hydrogen (electron) transport coenzyme for a wide variety of enzyme systems in both the "aerobic" and "anaerobic" phases of cell respiration. For example, it is coenzyme for lactic dehydrogenase, malic dehydrogenase and phosphoglyceraldehyde dehydrogenase.

$$\text{e.g. DPN} \quad \text{LACTIC ACID} \xrightleftharpoons[\text{Dehydrogenase}]{\text{Lactic}} \text{DPN-H}_2 + \text{PYRUVIC ACID}$$

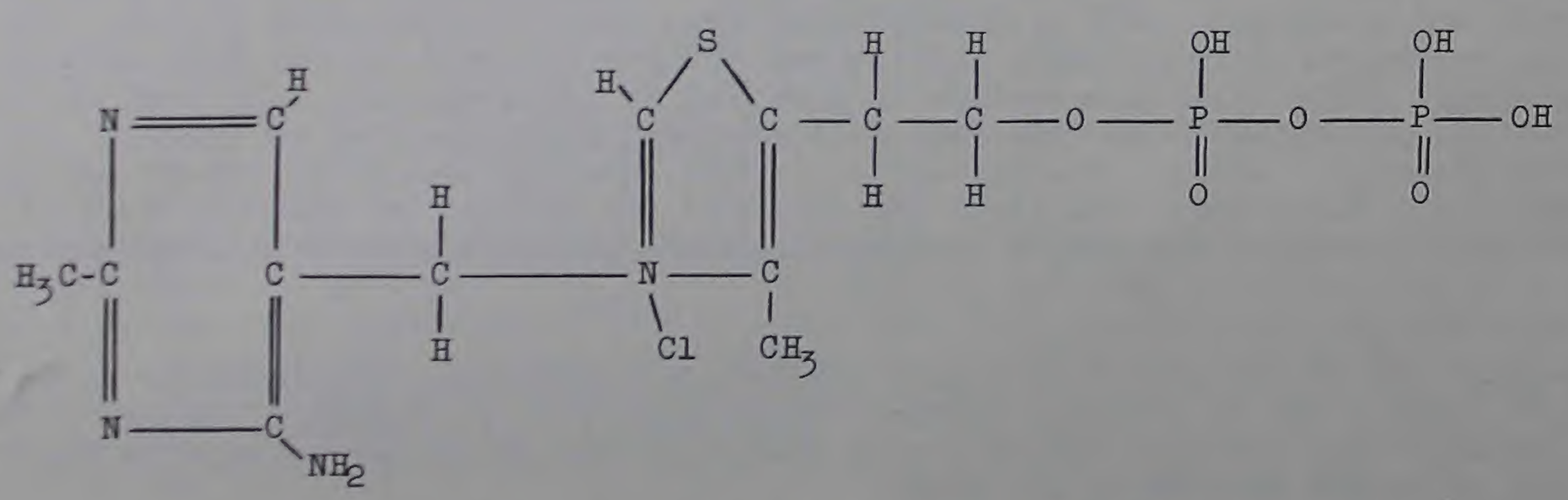
TPN functions have not been as extensively studied, but several TPN-linked dehydrogenases are known; for example, the glucose-6-phosphate dehydrogenase mentioned earlier, and isocitric acid dehydrogenase are both TPN-linked.

THIAMINE

Neuberg and Karczag (1911) found an enzyme in yeast which catalyzed the decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide. The decarboxylation of this and other α-keto acids has since assumed considerable importance in yeast and animal tissues. Auhagen (1932) showed that a coenzyme was necessary for this enzyme. It could be removed from yeast by washing with alkaline phosphate buffer. He also demonstrated its presence in animal tissues. Lohmann and Schuster (1937) isolated this coenzyme, which had become known as cocarboxylase, and demonstrated that it was thiamine pyrophosphate.

Besides yeast, Auhagen has reported the presence of cocarboxylase in liver, kidney, heart, and blood. None was found in muscle, though recently its presence in small amounts has been reported there. Its presence in rat brain and liver was observed by Simola. Tauber reports the presence of cocarboxylase in a large number of vegetables. In animal tissues the largest amounts are found in liver and kidney. The structure of cocarboxylase is represented in Formula III.

FORMULA III

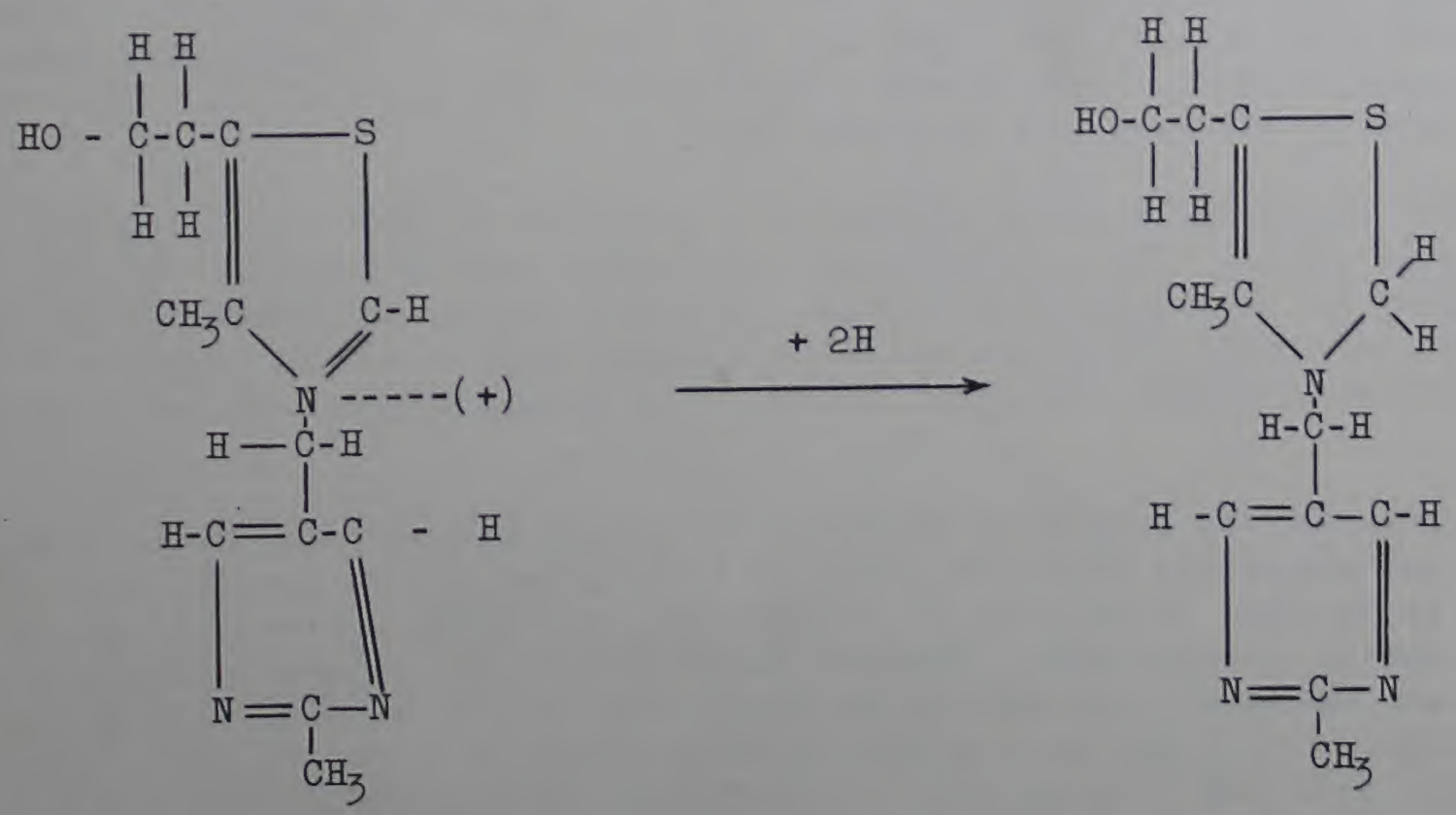


Thiamin pyrophosphate - cocarboxylase

As with inorganic pyrophosphate, one molecule of phosphate is readily removed by acid hydrolysis. It is split off by hydrolysis in 1.0 N HCl at 100° C. for 15 min. The other is more resistant. Alkaline oxidation with ferricyanide produces a phosphorus-containing product with blue fluorescence in ultra-violet light, (thiochrome reaction). The absorption spectrum shows 2 bands with maxima at 245, and 260 mμ which is the same as given for thiamin. Cocarboxylase is an amphoteric substance. Cataphoretic experiments with the Theorell apparatus show a migration toward the cathode at a pH more acid than 3.8, and toward the anode at pH's above 3.8.

Lohmann's discovery that cocarboxylase contained thiamin was of great interest because it offered a definite function for vitamin B_1. A decrease in the cocarboxylase content of the tissues had previously been observed in B_1 - deficient animals. Lohmann and Schuster (1937) showed that cocarboxylase could replace vitamin B_1 in the treatment of B_1 deficient pigeons, and that it was active in the catatorulin test of Peters. Pyruvic acid accumulates in the blood and brain in vitamin B_1 deficiency. Disappearance of pyruvic acid and normal carbohydrate metabolism is restored upon addition of the vitamin (Peters 1936, 1937, Sherman and Elvehjem 1936). As cocarboxylase contains this vitamin, and definitely functions in the decarboxylation of pyruvic acid in yeast, the hypothesis was advanced that in animal tissues vitamin B_1 serves as a precursor for cocarboxylase and that it likewise functions in decarboxylating pyruvic acid. However, it has been shown that animal tissues and certain bacteria do not decarboxylate pyruvic acid directly like yeast, but instead oxidize it to acetic acid and CO_2, either by dismutation under anaerobic conditions (with 1 molecule being reduced to lactic acid) or directly. Lipmann (1937) finds that acetone preparations of L. Delbruckii can dehydrogenate pyruvic acid to acetic acid and CO_2, and yet not carry out direct decarboxylation, and regards cocarboxylase as the prosthetic group of the pyruvic dehydrogenating enzyme. The presence of free phosphate is necessary for the dehydrogenation; arsenate will work equally well. The dehydrogenation is independent of any dismutation. Lohmann and Schuster (1937) had suggested that cocarboxylase, as a prosthetic group, may combine with one protein in yeast and with others in other tissues, and it may be that in Lipmann's observations it is combined with a protein component forming a pyruvic acid dehydrogenase. In this connection it is of interest to note Lipmann's (1938) observations on the hydrogenation of thiamin. He found that by hydrogenation with sodium hydrosulfite, one molecule of thiamin took up 2 atoms of hydrogen, and concluded that the hydrogenation takes place at the double bond adjoining the quaternary nitrogen in the thiazole part of the molecule:

FORMULA IV



Thiamin

Reduced thiamin

During the reduction he observed a yellowish-green intermediate, suggestive of 2 step reduction. The reduction of thiamin by hydrosulfite was not reversible, inasmuch as sulfite from the hydrosulfide cleaves thiamin. In tissues, however, a reversible dehydrogenation of the thiazole ring is conceivable if the hydrogenated thiamin is stabilized, possibly by combination with a specific protein (dehydrogenase).

Of importance is the relationship between thiamin and cocarboxylase in animal tissues. Lipschitz, Potter, and Elvehjem (1938) have demonstrated the simultaneous existence of both compounds in tissues and suggest that the two compounds may exist in dynamic equilibrium. They have shown that the vitamin may be phosphorylated by brewer's yeast which has been washed free of cocarboxylase with alkaline phosphate buffer, provided hexosediphosphate, vitamin B_1 and certain thermostable factors present in boiled tissue extracts are present. This synthesis was blocked by iodoacetate and it was suggested that phosphorylation occurred as a result of the esterification of inorganic phosphate coupled with the energy derived from the dismutation between triosephosphate and pyruvic acid or acetaldehyde. Ochoa and Peters (1938) have objected to this work and have claimed that synthesis was not achieved by these methods. They have found that with enzyme preparations from baker's yeast there is considerable augmentation of carbon dioxide production from pyruvic acid as a substrate when thiamin is added to cocarboxylase, even in the presence of iodoacetic acid. They conclude that the increased carbon dioxide production noted in the experiments of Lipschitz et al. is due simply to activation of cocarboxylase present in the boiled tissue extract by the thiamin which is added.

The usual method of determination is that of Lohmann and Schuster (1937). 1 gram of dried yeast is well washed with warm M/10 Na_2HPO_4 solution, and with distilled water. The residue is suspended in 10 cc. of M/10 phosphate, pH 6.2. One cc. of this washed yeast suspension and 0.4 cc. of the cocarboxylase solution are placed in the main chamber of a manometric vessel. 0.3 cc. of a sodium pyruvate solution, pH 6.2, containing 5.0 mg. of pyruvic acid, and 0.1 mg. of Mg, as $MgCl_2$, are placed in the side arm. After temperature equilibrium, the contents of the side arm are poured into the vessel and the CO_2 production is observed. Sample figures taken from Lohmann and Schuster are: without addition of cocarboxylase 25 cmm. of CO_2 are produced in 30 minutes, - after addition of 1.0 microgram of cocarboxylase 151 cmm. of CO_2 are produced.

Westenbrink and Goudsmit (1938) have applied the thiochrome reaction to the determination of cocarboxylase and thiamin separately in animal tissues. The principle of the method is that the free thiochrome formed by oxidation of the thiamin by potassium ferricyanide in alkaline aqueous solution is extracted by isobutanol, whereas the thiochrome pyrophosphate formed from the cocarboxylase remains in the aqueous solution. Both solutions are irradiated with ultraviolet light and the intensity of fluorescence measured, from which the concentration of thiamin and cocarboxylase are calculated. Improvements in this method were made by Hennessy and Cerecedo (1939).

Auhagen's first preparation was a concentrate prepared by addition of lead acetate to dialyzed boiled yeast juice. The precipitated lead salts were decomposed by H_2S, and the filtrate fractionated by treatment with barium. A modification and extension of this preparation from boiled yeast juice was given by Auhagen with which he prepared a concentrate 200 times more active than the yeast juice. The cocarboxylase was not prepared in pure form.

Lohmann and Schuster prepared pure crystalline cocarboxylase from brewer's yeast. The cocarboxylase was extracted from the yeast by boiling water and precipitated from alkaline solution by barium. A mixture of dilute HNO_3 and H_2SO_4 was used to elute the coenzyme from the barium precipitate. Further purification was accomplished by precipitation with ethanol and methanol, adsorption on Frankonite KL and elution with pyridine, fractional precipitation with methanol-ether, precipitation with picric acid, precipitation with phosphotungstic acid and finally crystallization of the hydrochloride. 100 kg. of yeast yielded 700 to 800 mg. of the crystalline hydrochloride of cocarboxylase.

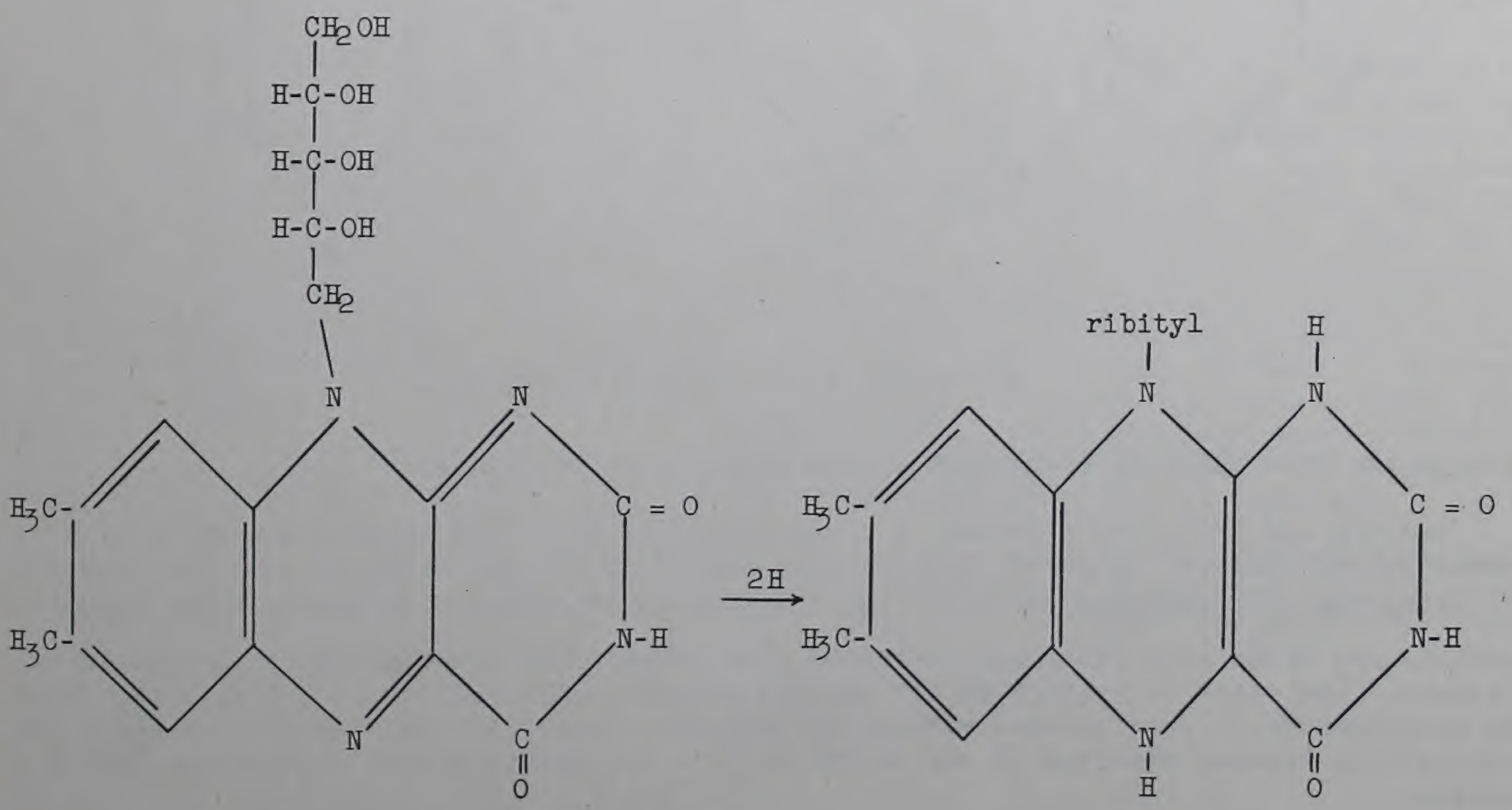
Cocarboxylase has been synthesized from thiamin by enzymatic and chemical methods. Kinnersley and Peters report its synthesis by live yeast. Euler and Vestin observed synthesis of cocarboxylase by the interaction of inorganic phosphate, adenosine triphosphate,

and thiamin in the presence of dried bottom yeast. Tauber reported its synthesis from thiamin and orthophosphate by means of an enzyme of the duodenal mucosa of the pig. Ochoa and Peters were unable to synthesize cocarboxylase with intestinal mucosa, but succeeded with liver. Stern and Hofer (1937) synthesized cocarboxylase by phosphorylation of thiamin with phosphorous oxychloride. Tauber, and Weijlard and Tauber (1938) report its chemical synthesis by the interaction of thiamin hydrochloride and a mixture of sodium pyrophosphate and phosphoric acid. They give a series of experiments to show that synthetic cocarboxylase as obtained from synthetic thiamin chloride is in every respect identical with cocarboxylase prepared from yeast. Ochoa (1939) made a study of cocarboxylase synthesis in animal tissues.

RIBOFLAVIN

Warburg and Christian (1932) isolated from yeast a yellow conjugated protein which they called the "yellow enzyme". By treatment of it with methyl alcohol they could resolve it into a colorless protein and a yellow coenzyme. Shortly afterward Kuhn et al (1935) isolated lactoflavin, the substance responsible for the yellow-green fluorescence of whey. The properties of this milk flavin were soon found to be identical with those of the prosthetic group from Warburg and Christian's "yellow enzyme". Both Kuhn (1935) and Karrer's (1935) laboratories independently determined the structure and accomplished the synthesis of the compound. It has since been found to be present in a wide variety of animal tissues, microorganisms and plants. It was found by Kuhn, Rudy and Weygand (1936) that the enzyme contained phosphate. They demonstrated that this was on the 5-position in the ribityl group. Formula V illustrates the structure of lactoflavin.

FORMULA V

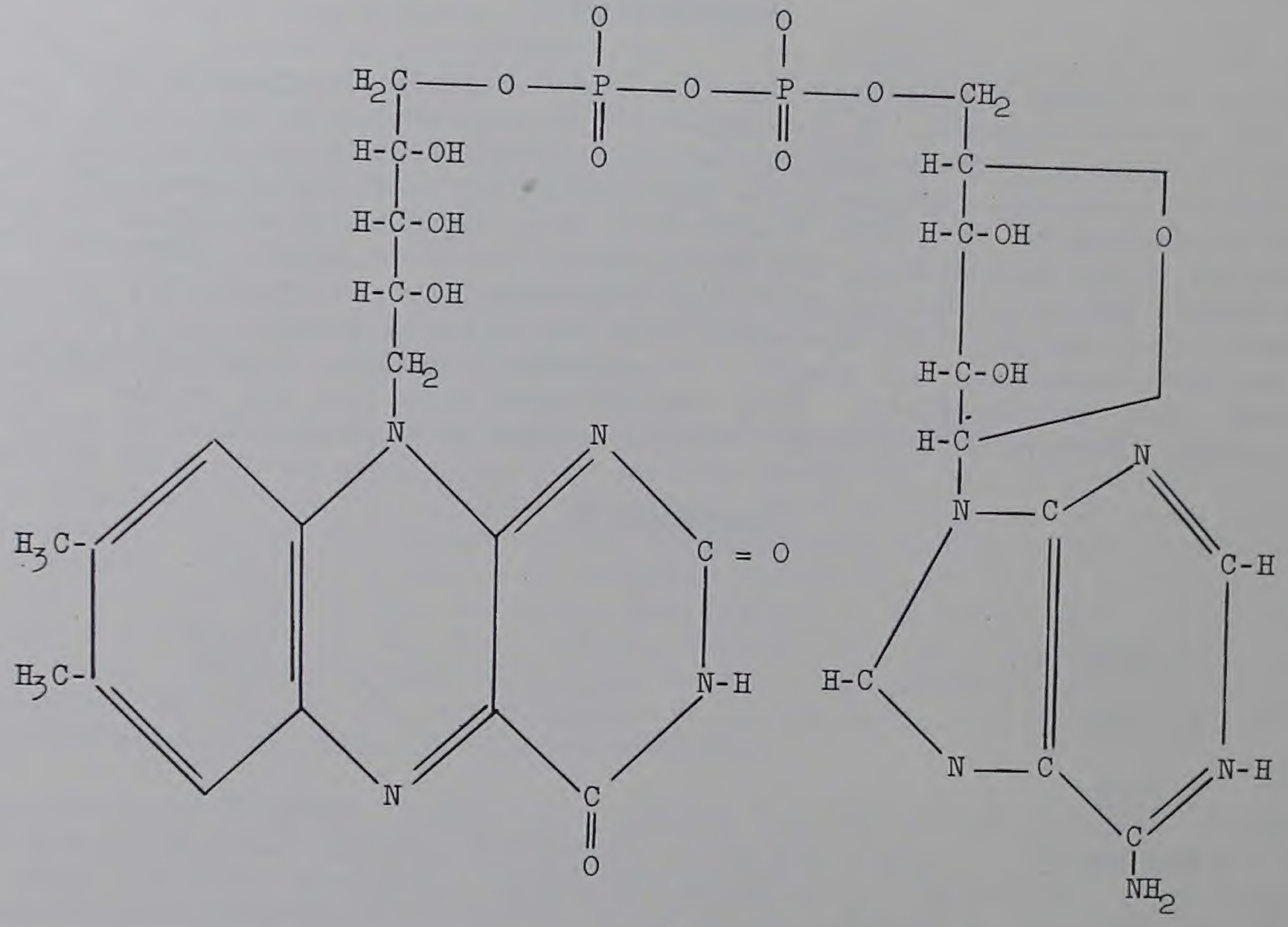


Alloxazine mononucleotide or Lactoflavin (6:7-dimethyl-9-(d-1-ribityl) isoalloxazin)

Dihydroalloxazine mononucleotide

Straub (1938) and Warburg and Christian (1938) discovered that the prosthetic group for D-amino acid oxidase was a flavin compound not identical with lactoflavin or its phosphate. The latter investigators established that it was a flavin adenine dinucleotide containing one molecule each of both flavin and adenine, two molecules of phosphoric acid and two molecules of sugar. The structure is illustrated in Formula VI.

FORMULA VI



Alloxazine adenine dinucleotide

This is the form which is found most widely distributed in tissues.

Warburg and Christian's method of preparation for this dinucleotide can be briefly summarized as follows: a yeast suspension is heated to 75° C., filtered, and the filtrate $\frac{2}{3}$ saturated with ammonium sulfate. The solution is extracted with phenol. The phenol extracts are mixed with ether and extracted with water. The dinucleotide is in the water extracts. The ether is evaporated off and the solution acidified to pH 2 with nitric acid. The dinucleotide is precipitated with silver and the silver salt decomposed with H_2S. The dinucleotide becomes adsorbed on the silver sulfide and can be eluted with dilute barium acetate.

The dinucleotide has a characteristic lemon-yellow color and a green fluorescence. It has absorption maxima at 275, 377 and 465 mμ. Hydrosulfite reduces it to the colorless or leuco-form, which can in turn be reoxidized by molecular oxygen. The reduction, in the case of the mononucleotide, is illustrated in Formula V. Biological activity of the dinucleotide is destroyed by short exposure to N alkali at room temperature or to N HCl at 100° C. The compound can be determined by measurement of its fluorescence. It is destroyed by exposure to ultra-violet light.

Ribose and ribityl groups in Alloxazine adenine dinucleotide are of D configuration instead of L as shown.

Flavoproteins can be split into the protein and flavin coenzyme by dialysis against dilute acid or by acidifying in the presence of large concentrations of ammonium sulfate. It is possible to recombine them without loss in activity when an excess of the flavin is used.

Thus far at least nine flavoprotein enzymes have been described. Since they will be covered in more detail in other chapters, it will suffice to merely mention them here as follows:

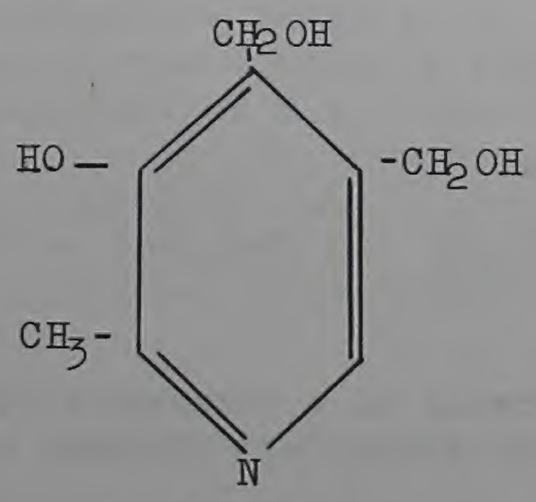
1. Warburg and Christian's (1932) "old yellow enzyme".
2. The d-amino acid oxidase identified by Krebs (1935).
3. The "new yellow enzyme" of Haas (1937).
4. The "Straub yellow enzyme" (1939) isolated from heart - probably identical with that of Haas isolated from yeast.
5. The "crossed yellow enzyme" of Warburg and Christian (1938a) which was the protein of the "old yellow enzyme" combined with alloxazine adenine dinucleotide.
6. "Xanthine oxidase" purified by Ball (1939).
7. "Fumaric dehydrogenase" discovered by Fischer and Eysenbach (1937).
8. "Aldehyde oxidase" isolated from liver by Gordon et al (1940).
9. "Cytochrome C reductase" isolated by Haas et al (1940).

PYRIDOXINE

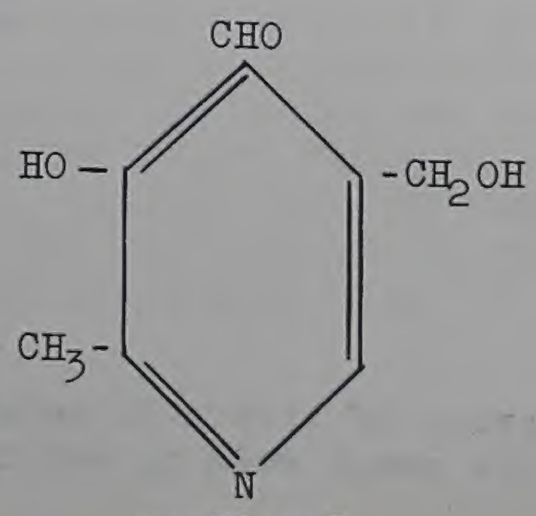
The function of another B-vitamin, referred to as vitamin B_6 and now known as pyridoxine, has also been shown to be in the structure of a coenzyme. As early as 1940, Gale reported studies of the tyrosine decarboxylation enzyme system in lactic acid bacteria. Bellamy and Gunsalus (1943) found that more pyridoxine was required for production of this enzyme in the bacteria than was required for maximum growth. Gunsalus and Bellamy (1944) suggested the function of pyridoxine might be as a coenzyme for this decarboxylase. Gale and Epps (1944a) prepared the specific enzyme and coenzyme for lysine decarboxylation and reported the codecarboxylase to be a pyridoxine derivative. Snell (1944) had found that the pyridoxine derivative which was active in stimulating the decarboxylase activity of lactic acid bacteria was pyridoxal. The structures are given in Formula VII. Simultaneous with Gale and Epps report was one by Gunsalus, Bellamy and Umbreit (1946), that the coenzyme of a tyrosine decarboxylase in bacteria was a pyridoxal phosphate. They demonstrated that pyridoxal itself was inactive, but that addition of adenosine triphosphate permitted formation of the active coenzyme by the bacterial preparation. Synthetic pyridoxal phosphate was also active. Gale and Epps had somehow missed the phosphate in the coenzyme and reported it free from phosphate. Further publications by Umbreit, Bellamy and Gunsalus (1945), Umbreit and Gunsalus (1945), Wood, Gunsalus and Umbreit (1947) and Lichstein, Gunsalus and Umbreit (1945) have established more widespread activities for this coenzyme. It has now been demonstrated that pyridoxal phosphate is the coenzyme for a variety of amino acid decarboxylases, for transaminases and for tryptophanase.

A claim by Karrer and Viscontini (1947) that a synthetic pyridoxal-3-phosphate prepared in their laboratory had the codecarboxylase activity was refuted by Gunsalus and Umbreit (1947). The exact position in the pyridoxal molecule where the phosphoric acid group is attached remains in some doubt.

FORMULA VII



pyridoxine



pyridoxal

The coenzyme can be prepared synthetically by the action of $POCl_3$ on pyridoxal in the cold. The active material can then be concentrated by precipitating with barium in alkaline solution to rid the solution of inorganic phosphorus and adding alcohol to precipitate the barium salt of the coenzyme. Preparation of the coenzyme from yeast is accomplished by extracting the dried yeast with dilute barium hydroxide, removing the barium from the extract with sulfate, acidifying to pH 3.0 and adding methanol to precipitate proteins. Then the coenzyme is precipitated from the supernatant by addition of barium acetate. The synthetic method is that of Umbreit, Bellamy and Gunsalus (1945). The preparation from yeast is that of Gale and Epps (1944b).

Umbreit, Bellamy and Gunsalus (1945) have also devised an assay method for measurement of pyridoxal and the codecarboxylase. It depends upon the use of a dried cell preparation made from bacteria grown in a pyridoxine deficient medium. This is incubated in Warburg respirometers with tyrosine and the test sample, with and without excess adenosine triphosphate added. The carbon dioxide liberation is measured. The carbon dioxide liberation without ATP added is a measure of codecarboxylase; that with excess ATP added is a measure of both codecarboxylase and pyridoxal.

PANTOTHENIC ACID

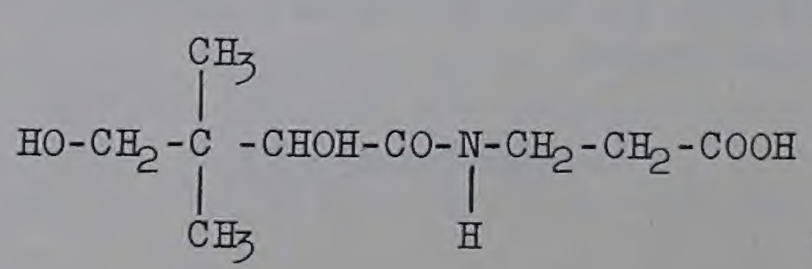
The reports concerning the function of pantothenic acid in metabolism are very recent, and consequently little information is available. Lipmann (1945) reported that the enzymatic acetylation of sulfanilamide required a heat-stable coenzyme. He has referred to this acetylation cofactor as "coenzyme A". Lipmann and Kaplan (1946) demonstrated that coenzyme A was also the coenzyme for the enzyme in nerve tissue which is involved in the synthesis of acetyl choline. It seems likely that this factor is a coenzyme for acetylation reactions in general. Lipmann et al (1947) were able to demonstrate that their coenzyme A contained approximately 10 per cent of pantothenic acid. Further studies demonstrated that in a series of preparations, the coenzyme A activity was correlated with pantothenic acid content.

A somewhat different function for the pantothenate coenzyme has been postulated by Dorfman et al (1942), who report that pantothenic acid is concerned in oxidation of pyruvic acid by certain bacteria. More recently McElroy and Dorfman (1948) present evidence which seems to localize their coenzyme effect on a reaction involved in the utilization of acetymethylcarbinol, formed by the bacteria in pyruvate oxidation. Thus there appears to be a multiple function for this coenzyme.

It would seem from the distribution of pantothenic acid, that the coenzyme is widely distributed. Lipmann and coworkers use pork liver as a source of their concentrates.

Since coenzyme A is not yet available in pure form, the chemical properties and structure are unknown. It is reported to contain phosphorus. Pantothenic acid has been synthesized and its structure is known.

FORMULA VIII



Pantothenic acid

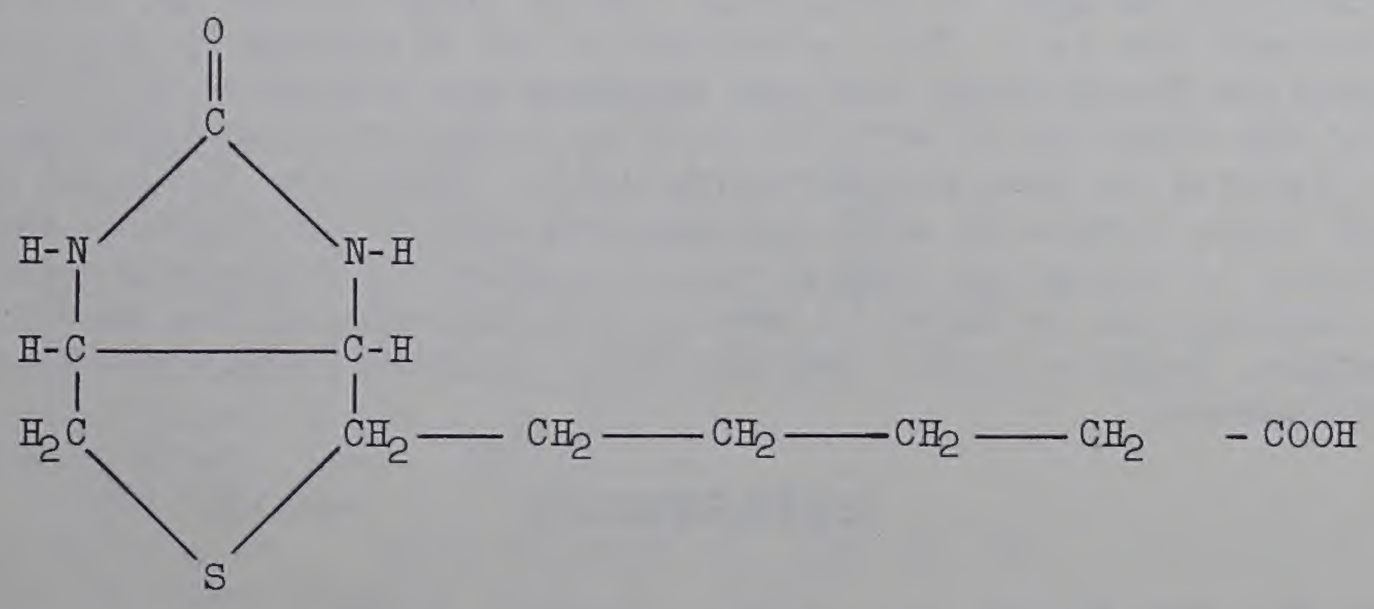
BIOTIN

The function of biotin in metabolism is undoubtedly as a component of a coenzyme or coenzymes. Its exact role is still in doubt. Koser, Wright and Dorfman (1942) reported

that growth of Torula cremoris was inhibited in biotin deficient media, and that the inhibition was largely prevented if adequate aspartic acid was supplied. Lardy, Potter and Elvehjem (1947) found that oxaloacetic acid could replace aspartic acid in this respect for L. arabinosus 17-5. They found that bicarbonate greatly stimulated growth in the presence of biotin but not in its absence. This made it seem likely that biotin was involved in the reaction of Werkman and Wood (1942) in which carbon dioxide is fixed by condensation with pyruvic acid to form oxaloacetic acid. Support for this concept from different data was provided by Shive and Rogers (1947) at the same time as that of Lardy et al. Lichstein and Umbreit (1947a) were able to obtain bacterial preparations which would enzymatically decarboxylate oxaloacetic acid to pyruvic acid and carbon dioxide. They were able to demonstrate the inactivation, presumably dissociation of a cofactor, of the enzyme by incubation of it at pH 4.0. On addition of biotin, activity was restored. This would seem to establish biotin as a coenzyme for the decarboxylation reaction (and reverse). Winzler, Burk and du Vigneaud (1944) had found that biotin-deficient yeast cells were markedly stimulated by biotin to take up ammonia. Lichstein and Umbreit (1947b) report that the deaminases of aspartic acid, serine and threonine may be resolved by incubation at pH 4.0 and that they too are reactiviated by biotin. Lardy and Robertson* have found in experiments with rats that C^{14} labelled bicarbonate injections result in C^{14} appearing in the adenine and guanine of the tissue nucleic acids. The fixation occurs to a much smaller extent in biotin deficient rats. It appears that biotin may be found to have multiple functions as a coenzyme.

It is not yet apparent whether biotin, as such, is all or a part of the coenzyme structure. Biotin itself has been synthesized and the structure is known.

FORMULA IX



Biotin

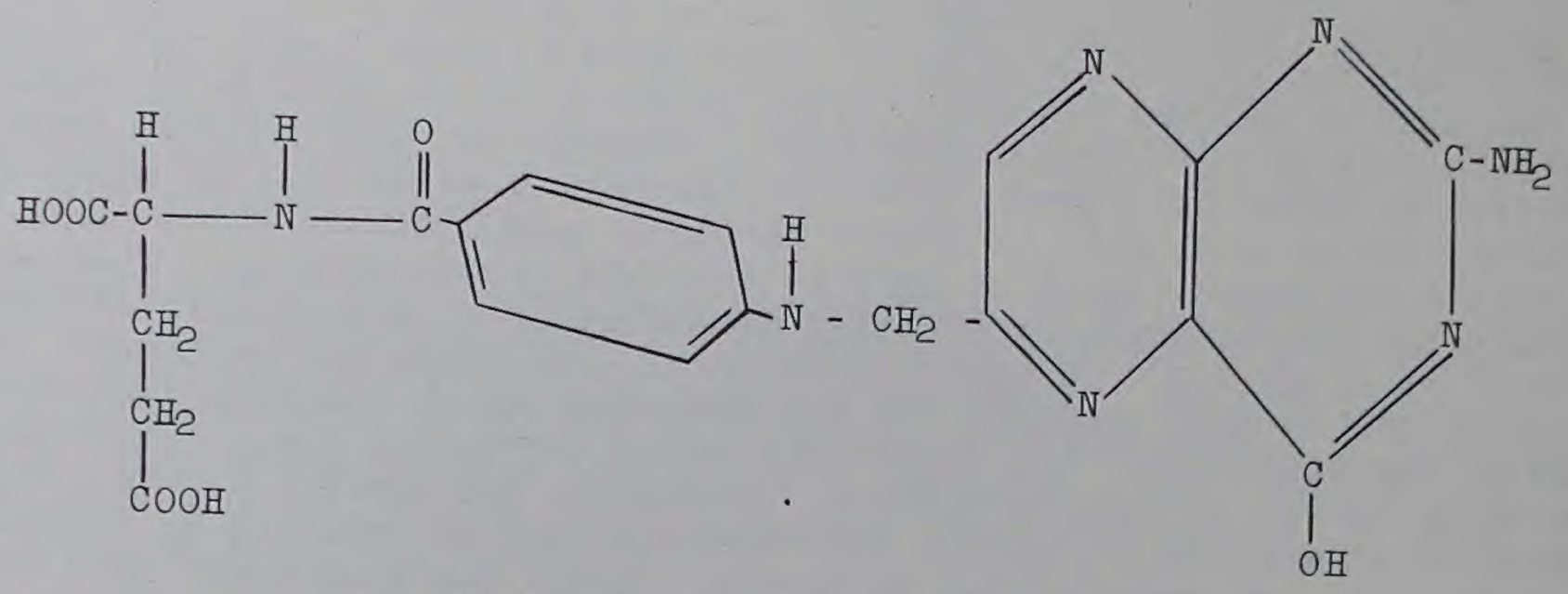
FOLIC ACID

Folic acid has recently been synthesized and the structure determined. p-aminobenzoic acid is a component of the structure. By inhibition analysis, Shive and Roberts (1946), demonstrated a requirement of p-aminobenzoic acid in purine synthesis by bacteria. When its presence in the folic acid structure became apparent, it was natural to assume that the necessity for p-aminobenzoic acid in purine synthesis was due to a folic acid requirement in purine synthesis. Shive et al (1947) demonstrated with a bacterial preparation that inhibition of purine synthesis with sulfonamides resulted in accumulation of an amine which could be construed as a precursor in purine synthesis. It lacked one carbon of the pyrimidine ring of the purine. Gordon et al (1948) prepared formyl folic acid and found it more active biologically than folic. By inference, it appears that the function of folic acid may be as coenzyme for the reaction in which the extra carbon is supplied to complete purine synthesis, this carbon being fixed in the pyrimidine ring via the formyl group of folic acid.

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*Lardy, H. A. and Robertson, P., personal communication.

FORMULA X



Folic acid (pteroyl glutamic acid)

ASCORBIC ACID AND GLUTATHIONE

Almost since its discovery, ascorbic acid has been suggested as a hydrogen carrier. It occurs in all animal tissues in amounts compatible with hydrogen transport or coenzyme function. The same comments can be made concerning glutathione. It is widely distributed in animal tissues too. Both compounds occur in the oxidized and reduced forms in tissues, and tissues are known to be able to oxidize or reduce them. There appears to be some interrelationship between the two. This relationship was discussed by Hopkins and Morgan (1936). Their possible function as hydrogen carriers was discussed by Potter (1940). Some indication of the function of ascorbic acid as cofactor in glutathione oxidation by mouse kidney was reported by Ames and Elvehjem (1946). However, it is not possible to classify either of these compounds as a coenzyme and define its function at present. It seems more reasonable to assign to them a role in control of oxidation-reduction potential of the tissues. Glutathione is known to prevent inactivation of the sulfhydryl groups of enzymes. For example, Rapkine (1938) demonstrated its value in activation of phosphoglyceraldehyde dehydrogenase.

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Chapter VI

DEHYDROGENASES

by

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INTRODUCTION

A dehydrogenase may be defined as an enzyme which activates certain hydrogen atoms of a metabolite so that they are capable of being transferred from the metabolite to a suitable acceptor. The first hydrogen acceptor may be oxygen or it may be some compound that occurs naturally in the tissues. In the study of dehydrogenases, one may use dyes with the correct oxidation-reduction potential in place of naturally occurring acceptors.

The catalytic process by which hydrogen is activated and removed from a metabolite is known as dehydrogenation, and it is one of the basic reactions by which many biological oxidations are accomplished. The concept of hydrogen activation was first advanced by Wieland (1922). The conditions necessary for activation and for hydrogen transport to take place have subsequently received much attention. Results of studies by many investigators have shown that dehydrogenases can act in the presence of oxygen and, under suitable conditions (with the exception of uricase), they are also active in the absence of oxygen. This is shown by the fact that under anaerobic conditions they decolorize dyes of the proper potential. This is important because it is the basis for distinguishing between dehydrogenases and oxidases, since the latter act only in the presence of molecular oxygen.

The decolorization that results from the reduction of dyes makes it possible to determine whether enzymes that can catalyze anaerobically the dehydrogenation of organic compounds such as glucose, ethyl alcohol, and succinic and malic acids are present in living tissues or in extracts of tissues. It should be pointed out, however, that in order for this reaction to take place, many of the dehydrogenases require the presence of coenzymes, such as the pyridine nucleotides, and other enzymes such as flavoproteins. These other factors occur naturally in tissues, although it is sometimes necessary to add them under experimental conditions in order to insure the action of the dehydrogenase.

Thunberg (1918, 1920) developed the simple dye technique, which has been used extensively for the detection and study of dehydrogenases in living tissues, including animal tissues, bacteria, yeasts, and in seed plants according to Berger and Avery (1943, 1944). The early work consisted mainly of adding various compounds or metabolites to these tissues in the presence of a suitable dye which was usually methylene blue (MB), and observing the rate of decolorization of the dye. Under standard anaerobic conditions the time required for complete decolorization is inversely proportional to the amount of enzyme present. Under aerobic conditions this reaction is also of value for quantitative estimations. The colorless leuco-base form of the dye that results from the action of the dehydrogenase is, in the presence of molecular oxygen, oxidized to the colored form. This consumption of oxygen can be measured as an index of the activity of the enzyme present. Results obtained by these procedures are limited in the sense that they do not answer many of the questions as to the mechanism of the action, the cofactors required, and other conditions necessary for the action of the different dehydrogenases.

Dehydrogenases in Reconstructed Systems

Much of the more recent work on the dehydrogenases has been directed toward their purification and isolation, and studies of them in reconstructed systems. Much work is usually required to reconstitute a system as to carriers and cofactors so that respiration will take place, as indicated by oxygen uptake and the oxidation of a particular metabolite. For example, the mechanism of the action of succinic dehydrogenase has been studied extensively. This system is now known to consist of succinate, succinic dehydrogenase, cytochrome c, cytochrome oxidase and oxygen. Apparently there is also an additional factor contained in tissues which is required to link the dehydrogenase to cytochrome c, but this factor has not been identified. Several other systems are quite well understood, as will be seen in the discussions of the individual dehydrogenases.

The question arises as to whether the reactions that are demonstrated in reconstructed systems also occur in intact cells or tissues. It should be pointed out (1) that the presence of dehydrogenases can be demonstrated within intact cells by the Thunberg method,

(2) that they are specific for particular hydrogen acceptors, (3) that the oxidation and reduction of cytochromes take place in living cells, and (4) that these same reactions and specificities are known to hold in the reconstructed systems. In view of these facts, it seems reasonable to assume that the reactions of reconstituted systems may also take place in intact living cells. It should be recognized, however, that the orientation of the various constituents within the cell probably results in a selection and direction of cellular reactions, and that these reactions may be redirected or even replaced by other reactions in accordance with the changing environment and requirements of the cell. This leads to the realization that results obtained should be treated cautiously when the activities of intact living cells are considered. On the other hand, it should be emphasized that work on the development and use of reconstituted systems has resulted in the accumulation of much important information on the conditions required for the action and the mechanism of action of dehydrogenases.

Specificity and Classification

The dehydrogenases have been found, as a rule, to be highly substrate-specific. The main exceptions are D-amino acid, L-amino acid, aldehyde, and the amine and xanthine dehydrogenases, which are group specific. The L-form of the substrate is the one usually activated, an outstanding exception being D-amino acid dehydrogenase which activates only D-amino acids. Furthermore, the dehydrogenases are quite specific as to the first acceptor of the activated hydrogen. There are, however, some interesting exceptions. In some cases it appears that the first hydrogen acceptor specificity depends on the kind of tissue with which the enzyme is associated, although the reason for these differences is unknown. Thus glutamic acid dehydrogenase of yeast and Escherichia coli (Bacillus coli) is specific for TPN (triphosphopyridine nucleotide, coenzyme II) and that of plant tissues for DPN (diphosphopyridine nucleotide, coenzyme I, cozymase), while the enzyme of animal tissues uses DPN and possibly also TPN. At least glutamic dehydrogenase of animal tissues, in the presence of α-ketoglutarate and ammonia, catalyzes the oxidation of reduced TPN as well as reduced DPN. Lactic dehydrogenase of animal tissues is DPN-specific and that of yeast is cytochrome c-specific. Similarly there are DPN- and cytochrome c-specific α-glycerophosphate dehydrogenases. Both of these are found in animal tissues.

The first acceptor of the activated hydrogen of the metabolite is also important from the standpoint of classification. On this basis the better known dehydrogenases are divided into two main groups. The first group contains those enzymes which activate hydrogen to react directly with oxygen. They are known as aerobic dehydrogenases. The second group includes the enzymes which activate hydrogen to react directly with a carrier. These are known as anaerobic dehydrogenases. The latter is the larger group and is divided into two sub-groups, depending on whether the first electron acceptor is a component of the cytochrome system, or a pyridine nucleotide coenzyme. There is a third group which consists of unclassified dehydrogenases whose mechanisms of action are less well understood. This is essentially the classification given in the first edition of Respiratory Enzymes (Elvehjem and Wilson, 1939) and in the recent review by Potter (1948). The dehydrogenases are classified according to this scheme in Table 1. Information on the better known unclassified dehydrogenases is given in Table 5.

The individual dehydrogenases are named according to the substrate on which they act. As Potter (1948) has pointed out, this has led to some confusion concerning the nomenclature of the aerobic group. Because one substrate is oxygen, they have often been designated as oxidases, such as D-amino acid and xanthine oxidases. However, because the enzymes of this group activate their substrates to react with dyes in the absence of molecular oxygen, they have also been referred to as dehydrogenases, and they are so classified in Table 1. Their natural hydrogen acceptor is oxygen, and the hydrogen peroxide formed is decomposed to hydrogen and oxygen by catalase.

TABLE I

Classification of Dehydrogenases According to Substrate and Hydrogen Acceptor

General Class	Name	Substrate	Hydrogen Acceptor
Aerobic Dehydrogenases	Xanthine dehydrogenase	Xanthine	Oxygen
	D-Amino acid dehydrogenase	D-Amino acids	Oxygen
	L-Amino acid dehydrogenase	(L-Amino acids (L-Hydroxy acids	Oxygen
	Glycine dehydrogenase	Glycine	Oxygen
	Uric acid dehydrogenase (uricase)	Uric acid	Oxygen
	Aldehyde dehydrogenase	Aldehydes	Oxygen
	Amine dehydrogenase	Monoamines	Oxygen
	Diamine dehydrogenase	Diamines	Oxygen
Anaerobic Dehydrogenases Cytochrome-linked	Succinic dehydrogenase	Succinic acid	Cytochrome c
	α-Glycerophosphate dehydrogenase	α-Glycerophosphoric acid	Cytochrome c
	Lactic dehydrogenase (yeast)	Lactic acid	Cytochrome c
	Choline dehydrogenase	Choline	Cytochrome c
Pyridine-nucleotide-linked	Malic dehydrogenase	Malic acid	DPN or TPN (21:1)
	Lactic dehydrogenase (animal tissues)	Lactic acid	DPN or TPN (170:1)
	β-Hydroxy butyric dehydrogenase	β-Hydroxy butyric acid	DPN
	Glutamic dehydrogenase	Glutamic acid	DPN or TPN
	Isocitric dehydrogenase	Isocitric acid	TPN
	Glucose-6-phosphate dehydrogenase	Glucose-6-phosphoric acid	TPN
	Triosephosphate dehydrogenase	Triosephosphoric acid	DPN
	α-Glycerophosphate dehydrogenase	α-Glycerophosphoric acid	DPN
	Alcohol dehydrogenase	Ethyl alcohol	DPN
	Glucose dehydrogenase	Glucose	DPN
	Formic dehydrogenase	Formic acid	DPN
Unclassified	Pyruvic dehydrogenase	Pyruvic acid	
	Hydrogenase	Molecular hydrogen	Oxygen

Mechanism of Dehydrogenase Action

Compounds of various kinds are oxidized as a result of being activated by dehydrogenases. α-Amino acids, ethyl alcohol, and lactic, malic, isocitric and succinic acids, as well as certain other compounds, are oxidized by the direct removal of two hydrogens. This type of oxidation is represented by the following equation for succinic acid:

$$\underset{\text{Succinic acid}}{HOOC\text{-}CH_2\text{-}CH_2\text{-}COOH} + \tfrac{1}{2}O_2 \longrightarrow \underset{\text{Fumaric acid}}{HOOC\text{-}CH{=}CH\text{-}COOH} + H_2O.$$

It is not so evident, however, that the oxidation of aldehydes, and α-keto acids, such as pyruvic, is accomplished by the mechanism of dehydrogenation. In the case of aldehydes,

oxygen is added to the molecule, even though the reaction takes place in the absence of oxygen. Wieland (1922) explained this by assuming the presence of a small amount of hydrated aldehyde in the reaction mixture. This provides the basis for the presently accepted explanation of the dehydrogenation of aldehydes and α-keto acids. They first undergo hydration at the respective aldehyde and keto groups, and this is followed by dehydrogenation in the presence of the enzyme, providing a suitable hydrogen acceptor is present. Decarboxylation also occurs in the case of pyruvic acid. The following equations, in which methylene blue (MB) is used as the acceptor, are representative of this type of reaction:

$$\underset{\text{Glucose}}{CH_2OH\text{-}(CHOH)_4\text{-}CHO} + H_2O \longrightarrow \underset{\text{Glucose hydrate}}{CH_2OH\text{-}(CHOH)_4\text{-}CH(OH)_2} \xrightarrow{\text{Dehydrogenase + MB}} \longrightarrow \underset{\text{Gluconic acid}}{CH_2OH\text{-}(CHOH)_4COOH} + MB.H_2$$

$$\underset{\text{Pyruvic acid}}{CH_3\text{-}CO\text{-}COOH} \longrightarrow \underset{\substack{\text{Pyruvic acid}\\ \text{hydrate}}}{CH_3\text{-}C(OH)_2\text{-}COOH} \xrightarrow{\text{Dehydrogenase + MB}} \underset{\text{Acetic acid}}{CH_3\text{-}COOH} + CO_2 + MB.H_2.$$

The oxidation of xanthine and hypoxanthine are other examples of hydration followed by dehydrogenation.

More recent investigations reported by Lipmann (1939a) show that inorganic phosphate may take the place of water in the "hydration" process. As a result of oxidation, the phosphate becomes fixed as acyl phosphate. The generation and utilization of energy-rich phosphate bonds are discussed elsewhere. The reaction for pyruvic acid is represented as follows:

$$\underset{\text{Pyruvic acid}}{CH_3\text{-}CO\text{-}COOH} \xrightarrow{H_3PO_4} \underset{\substack{\text{Pyruvic acid}\\ \text{phosphate hydrate}}}{CH_3\text{-}C(OH)OPO_3H_2\text{-}COOH} \xrightarrow{\text{Dehydrogenase}} \underset{\text{Acetyl phosphate}}{CH_3\text{-}CO\text{-}OPO_3H_2} + 2H + CO_2$$

Another example of this type of dehydrogenation involves the addition of phosphate to the aldehyde group of phosphoglyceric aldehyde before it is oxidized to 1,3-diphosphoglyceric acid.

When the activated hydrogen of a specific substrate does not react directly with oxygen, as is the case with anaerobic dehydrogenases, the reaction becomes more complicated because carriers and other enzymes, in addition to the activating dehydrogenase, take part in the reaction. The entire mechanism by which the hydrogen is transported is spoken of as a system, such as the succinoxidase system or the malic dehydrogenase system. Each of the hydrogen atoms from the substrate loses an electron, which results in the formation of hydrogen ions. Both the electrons and the hydrogen ions take part in the process of hydrogen transport. The transfer of electrons is the common factor of biological oxidation by dehydrogenation, as is the case for all oxidations and reductions.

The hydrogen ions of the cytochrome-linked dehydrogenase systems, such as the succinoxidase, come into equilibrium with the buffer system of the reaction medium, whereas the electrons are carried over the cytochrome system by alternate reduction and oxidation of cytochrome c. In the presence of cytochrome oxidase, electrons from reduced cytochrome c unite with oxygen atoms to form oxygen ions, which then unite with hydrogen ions from the buffer system to form water. Thus the mechanism of dehydrogenation in systems of this kind is more complicated than is indicated when the overall reaction is written in the simple form of direct dehydrogenation with the formation of water.

Dehydrogenase systems in which the cytochrome system functions are inhibited by cyanide, but are active in the presence of cyanide providing an autoxidizable dye of correct oxidation potential is present. This is due to the fact that the cytochrome oxidase of the system is the site of action of the cyanide and a dye which is not affected by cyanide can take the place of the oxidase in the reaction.

DPN and TPN as Hydrogen Acceptors

The first hydrogen acceptors of the pyridine nucleotide dehydrogenases are DPN and TPN. Some dehydrogenases react with DPN and others with TPN. In the presence of the dehydrogenase, the coenzyme is reduced as it receives the activated hydrogen from the substrate. Then the hydrogen of the reduced coenzyme is activated by 'yellow enzymes' to react either with oxygen, with an autoxidizable dye such as methylene blue, or with cytochrome c.

Some oxidizing agents not found naturally in tissues, such as ferricyanide, are rapidly reduced in the presence of certain dehydrogenases and their respective substrates. Quastel and Wheatley (1938) showed that this is true for the cytochrome-linked succinic and choline dehydrogenases. The reduction of ferricyanide is also catalyzed by pyridine-nucleotide-linked dehydrogenases such as lactic, malic and glutamic. Dixon and Zerfas (1940) found that the hydrogen acceptors for alcohol and malic dehydrogenases of yeast fall into three groups on the basis of whether or not they require the presence of DPN and/or flavoprotein. Most dyes and cytochrome require both these substances; benzoquinone, o-quinone, phenazine dyes and potassium ferricyanide require DPN but not flavoprotein; whereas alloxan, iodine, hydrogen peroxide and dibromophenolindophenol do not require either DPN or flavoprotein. The reduction of the compounds in the latter group in the presence of the dehydrogenases and their respective substrates, in the absence of coenzyme, indicates that the dehydrogenases are complete enzymes and do not require the coenzymes as prosthetic groups in order to activate their substrates.

The combination of coenzymes, such as DPN, with dehydrogenases such as the lactic enzyme, is temporary, due to the slight affinity between the two substances. Although the linkage is weak and temporary in nature, the coenzymes function with the dehydrogenases in essentially the same capacity as firmly bound prosthetic groups such as, for example, hematin in the case of peroxidase. Baldwin (1947) states that the difference between coenzyme and prosthetic group in relation to the oxidation of their substrates is one of degree rather than of kind. It is of relatively little importance whether or not coenzymes such as DPN are considered as prosthetic groups of dehydrogenases providing the functional significance of the various parts of the particular system is clear. The reader is referred to Baldwin (1947), Schlenk (1945), and Ochoa (1946) for further discussion of this subject.

Dehydrogenases have been found to catalyze reversible reactions, the equilibrium points of which depend on the free energy of the reactions. The conditions required for the demonstration of reversibility are known in many cases. For example, Green and Brosteaux (1936) showed that lactic dehydrogenase, in the presence of DPN and lactate, oxidizes the lactate to pyruvate and reduces the DPN. That the reverse reaction is catalyzed was shown by the fact that reduced DPN is reoxidized and pyruvate is reduced to lactate in the presence of the dehydrogenase. The point of equilibrium lies far to the side of lactate. For this reason certain of the dehydrogenases have sometimes been called reductases. The equilibrium state of a reversible system of this kind can be expressed either in terms of the equilibrium constant or the oxidation-reduction potential.

The Role of Intermediary Carriers

In cellular metabolism it is important that certain of the dehydrogenase systems are coupled through intermediary carriers. This makes it possible for metabolic oxidations and reductions to take place in cells and tissues under anaerobic conditions. These coupled reactions were once thought to be due to the influence of a single enzyme which was designated as a mutase. Later it was found that reactions of this kind are the result of the action of two dehydrogenases which are coupled through a common carrier. These coupled oxidation-reduction reactions have been studied intensively by Green and his coworkers. They showed that for extracts the coupling takes place only in the presence of a reversibly oxidizable and reducible substance such as methylene blue. For example, lactate is oxidized to pyruvate in the presence of lactic dehydrogenase and methylene blue, and the reduced dye is reoxidized in the presence of fumarate and succinic dehydrogenase since the latter catalyzes the reduction of fumarate to succinate. An overall equilibrium is established between these reactants in the presence of the two dehydrogenases.

DPN can act as an intermediary between two dehydrogenases, providing both the enzymes are capable of acting with this particular pyridine nucleotide. In the presence of β-hydroxybutyric and alcohol dehydrogenases, β-hydroxybutyric acid is oxidized to acetoacetic acid, and acetaldehyde is reduced to alcohol. According to Green (1940) this reaction goes almost to completion before equilibrium is established. The reaction is represented as follows: β -Hydroxybutyric acid + Acetaldehyde $\rightleftharpoons$ Acetoacetic Acid + Alcohol.

The linkage of the triosephosphate and lactic dehydrogenase systems through DPN is described in the chapter on GLYCOLYSIS. Thus the coenzymes are effective intermediaries for the transport of hydrogen between different anaerobic dehydrogenase systems in addition to the important part they play in the transport of hydrogen to oxygen. It should be pointed out, however, that the dehydrogenase system, which catalyzes the oxidation of reduced coenzyme, must have a higher oxidation-reduction potential than the system which reduces the coenzyme.

Recently Mehler, Kornberg, Grisolia and Ochoa (1947) reported results which show that malic and lactic dehydrogenases react to a small extent with TPN and are not exclusively specific for DPN as was previously supposed. This has significance in that it provides the basis for an explanation of the coupled reaction between isocitrate and oxalacetate on the one hand, and malate and oxalsuccinate on the other. Isocitric dehydrogenase is TPN specific. The ratio of the activity of malic dehydrogenase for DPN and TPN, and similar data for the lactic enzyme, are given in Table 1, and the results for different tissues are found in Table 2.

TABLE II

Reaction of Malic and Lactic Dehydrogenases with Coenzymes*

Dehydrogenase	Source	Coenzyme (in optimal concentration)	Initial Rate (moles x 10^{-9} per cc. per min.)	Ratio DPN/TPN
Malic	Pig heart (pure)	DPN	2.40	22
	" "	TPN	0.11	
	Pig heart (crude)	DPN	2.70	16
	" "	TPN	0.17	
	Pigeon liver (crude)	DPN	4.70	14
	" "	TPN	0.34	
Lactic	Beef heart (crystalline)	DPN	6.80	170
	" "	TPN	0.04	
	Pigeon liver (crude)	DPN	3.04	101
	" "	TPN	0.03	

*These data are taken from a report by Mehler, A. H., Kornberg, A., Grisolia, S., and Ochoa, S. on "The specificities of some dehydrogenases toward pyridine nucleotides." Federation of American Societies for Experimental Biology Meetings, Chicago, Ill., May 1947. An abstract of this report is published in Federation Proceedings, 6, 278, 1947.

The following discussion of the individual dehydrogenases does not include all those of any one group, but rather several representative ones from each group. Their discovery and history are considered briefly, but more consideration is given to their source, preparation and purification; their quantitative determination and specificity; the conditions necessary for the reaction catalyzed to take place; their chemical and physical properties; and their inhibition. These factors are usually discussed in this order, but in the longer sections several of them are discussed under a single heading. The formulae given in the equations are written in the form of the free acids. These various acids occur in living cells and reconstructed systems in the form of salts such as potassium and sodium salts. Reference is made to many of the important papers on dehydrogenases, the review by Elliott (1941), and the monograph by Green (1940). The second edition of Enzymes by Sumner and Somers (1947) was helpful as a starting point in summarizing certain of the more recent contributions.

AEROBIC DEHYDROGENASES

XANTHINE DEHYDROGENASE

(Xanthine Oxidase, Schardinger Enzyme, Aldehydrase)

When formaldehyde and methylene blue are added to fresh milk in the absence of oxygen, the dye is reduced to the leuco-form and the blue color disappears (Schardinger, 1902). Wieland (1914) showed that aldehyde is oxidized aerobically in presence of milk. Morgan, Stewart and Hopkins (1923) found an enzyme in milk which catalyzes the oxidation of hypoxanthine and xanthine to uric acid. They confirmed the reports of other workers that many animal tissues also contain an enzyme which converts these purines to uric acid.

There has been much controversy as to whether the purines and aldehydes are activated by the same or by different enzymes. Work done mainly by Booth (1935, 1938a), and by Dixon and Keilin (1936) established that only one enzyme is involved. They showed that two enzymes cannot be separated by ordinary fractionation procedures, and that non-identity cannot be shown by chemical and physical treatments, or by inhibitors such as cyanide. The distribution of the two activities in different tissues and in milk are the same. They appear simultaneously during embryonic development, and inhibition by uric acid is the same for both purines and aldehydes. The catalytic action is independent of coenzymes and carriers and is effective with the same oxidants. Finally, when purine and aldehyde are added together in optimum concentrations no additive effect is obtained.

According to Morgan (1926) the occurrence of xanthine dehydrogenase in nature is very irregular. It is present in milk and in the livers of most animals, although it is absent from the livers of the dog, hedgehog and pigeon, and from heart and skeletal muscle. It is found in the kidneys of the cow, rat and birds, but is absent in other species tested. Liver is the only tissue of man in which it is present. It accumulates in the brain of mice with yellow fever encephalitis (Bauer, 1947).

Methods of Preparation and Determination

A number of methods for preparing xanthine dehydrogenase have been reported. Milk and liver have been used as sources. Dixon and coworkers (1924a, 1926) concentrated it from milk by ammonium sulfate precipitation, by treatment with charcoal, by adsorption on kaolin at pH 5.0 and by elution with dilute bicarbonate solution.

A more effective method of purification was reported by Ball (1939). The fat globules of fresh whole milk on which the enzyme is adsorbed were separated by centrifuging. On treatment with 0.2 M Na_2HPO_4 the enzyme was obtained in the aqueous layer. This extract was digested with pancreatic lipase and after treatment with $CaCl_2$, followed by centrifuging, a clear yellowish solution of the enzyme was obtained. Concentration was effected by use of ammonium sulfate. The enzyme, which precipitated between 33 and 42 per cent saturation, dissolved to give a clear brownish solution. It was 500 times more active per unit of dry weight than the starting material. The final product gave a Q_{O2} of 270, and

was stable for several months when precipitated and kept under ammonium sulfate at 20°C. The isoelectric point was pH 6.2, and the molecular weight was calculated to be 74,000 (Ball, 1939).

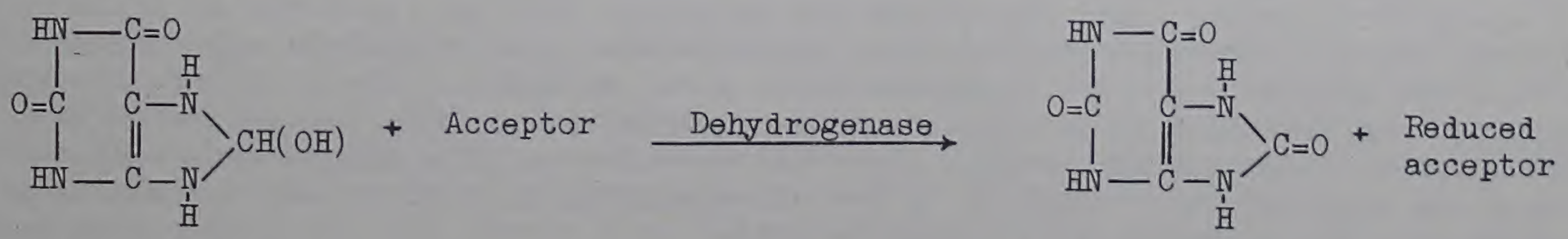
Corran, Dewan, Gordon and Green (1939) obtained a flavoprotein from milk that catalyzed the oxidation of hypoxanthine, aldehydes and reduced DPN. The milk was saturated with NaCl at 30°C., and the enzyme was recovered from the filtrate by precipitation with ammonium sulfate. Further purification was effected by dissolving the precipitate in phosphate buffer and reprecipitating with ammonium sulfate, then with alcohol and adsorption on alumina gel. The enzyme was eluted from the gel, and the final product was precipitated at a concentration of 38 to 45 per cent saturation with ammonium sulfate. The overall yield was 1.8 per cent, and the preparation was about one thousand times more active per milligram of dry weight than the milk. Incubation with cyanide and drying inactivated the hypoxanthine and aldehyde activities without affecting the DPN activity or the spectrum and chemical properties of the flavoprotein.

Ball (1938a, 1939) reported that solutions of his preparation show an absorption band between 400 and 500 mμ. This band fades but does not entirely disappear when hypoxanthine is added in the absence of air or oxygen, but reappears when air is readmitted to the system. If the spectrum of the reduced enzyme is subtracted from that of the oxidized form, a spectrum is obtained which is similar to that of the first yellow enzyme in that absorption bands are found at 370 and 465 mμ. The prosthetic group can be removed from the protein by acidification, by heat and by treatment with alcohol. Partial inactivation of the purified enzyme results from prolonged dialysis, but the activity is restored by the addition of the group removed during dialysis. The prosthetic group of xanthine dehydrogenase is alloxazine adenine dinucleotide (Warburg and Christian, 1938; Ball, 1938a); this was confirmed by Corran et al. (1939).

Ball (1939) used oxygen consumption as an index of xanthine dehydrogenase activity. The reaction mixture consists of 1 ml. of 0.1 M phosphate buffer of pH 7.2 plus enzyme solution to make 3 ml. The substrate was 0.2 ml. of 0.05 M hypoxanthine. Corran et al. (1939) used the Thunberg method to study their purified enzyme. Axelrod and Elvehjem (1941) determined the activity in homogenates of liver tissue by measuring the oxygen consumption manometrically.

Specificity and Action

Xanthine dehydrogenase catalyzes the oxidation of the hydrated forms of hypoxanthine and xanthine to xanthine and uric acid respectively. The reaction for xanthine is as follows:

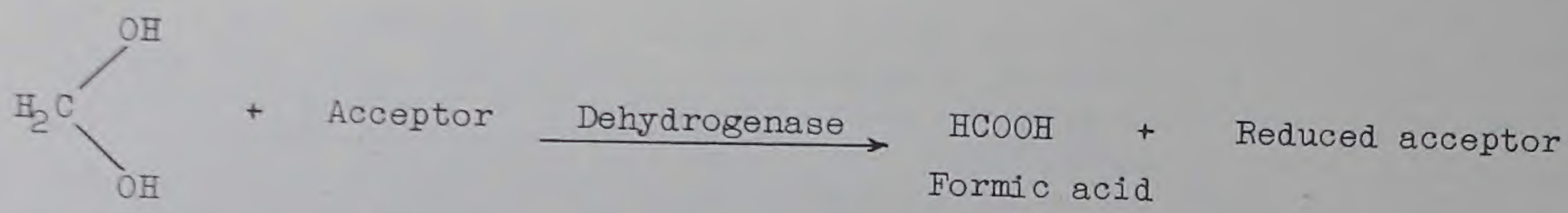


Xanthine (hydrate) Uric acid

The enzyme catalyzes the oxidation of purines such as 6,8-dihydroxy-purine and 2-thioxanthine (Coombs, 1927). Booth (1938a) showed that 6-amino-8-hydroxy and other purines are also oxidized. According to Dixon and Lemberg (1934) the purified enzyme does not oxidize purines which are combined to form complex compounds, but other enzymes in crude preparations liberate hypoxanthine so that oxidation takes place.

Xanthine dehydrogenase is rather unspecific in the sense that many substances, besides oxygen and methylene blue, will oxidize hypoxanthine in the presence of the enzyme (Dixon, 1926). These include hydrogen peroxide, indigo dyes, indophenol, alloxan, chlorates, iodine, nitrates, nitrobenzene, permanganate and quinone.

Booth (1938a) and Ball (1939) showed that the xanthine enzyme activates both aliphatic and aromatic aldehydes. The action of the purified enzyme on formaldehyde is represented as follows:



Formaldehyde (hydrate)

The initial rate of oxidation of purines and aldehydes is about the same, but it decreases rapidly in the case of the aldehydes. Hydrogen peroxide is formed when hypoxanthine, xanthine and aldehydes are oxidized in the presence of oxygen and the dehydrogenase (Thurlow, 1925). Dixon (1925) and Dixon and Keilin (1936) found that the enzyme is inactivated by the hydrogen peroxide unless it is decomposed by catalase or by the peroxidase-like action of methemoglobin. Keilin and Hartree (1945) confirmed the report that hydrogen peroxide is produced by the xanthine system. They also showed that the addition of ethyl alcohol to a primary oxidizing system of this kind that contains catalase, doubles the oxygen consumption. This is because hydrogen peroxide formed in the primary reaction is used secondarily for the coupled oxidation of alcohol.

Xanthine dehydrogenase has a high affinity for hypoxanthine and xanthine. The full rate of reaction is obtained with concentrations of purine of less than 3×10^{-5} M (Dixon and Thurlow, 1924b). When this concentration is increased the oxidation rate is decreased. This appears to be the result of excessive adsorption of the substrate on the enzyme which prevents access to the methylene blue. Booth (1938b) obtained maximum activity with 1.5×10^{-4} M hypoxanthine. The affinity of the enzyme for aldehydes is much lower, since it required 0.06 M acetaldehyde to give maximum activity. The affinity is greater for aromatic than for aliphatic aldehydes.

Green (1934) demonstrated (1) that the xanthine dehydrogenase system is reversible, (2) that the oxidation of hypoxanthine to uric acid is a two-step, reversible oxidation, and (3) that there are two equivalents involved in each step.

In the presence of purines and in the absence of oxygen, xanthine dehydrogenase causes a slow mutase action in that one molecule of xanthine is oxidized to uric acid whereas another is reduced to hypoxanthine (Green, 1934). According to Booth (1935) the enzyme is capable of catalyzing a reaction between uric acid and aldehydes to give hypoxanthine and the corresponding acids. When the enzyme was incubated with uric acid and salicylaldehyde, almost all the uric acid disappeared; when acetaldehyde, propionaldehyde and furfuraldehyde were used the uric acid did not disappear to as great an extent. Booth called this <u>mixed dismutation</u> and presented it as evidence that aldehydes and purines are activated by a single enzyme. Dixon and Lutwak-Mann (1937) showed that xanthine dehydrogenase does not catalyze the dismutation of aldehyde to the corresponding acid and alcohol; instead, it catalyzes only the oxidation of aldehyde to acid.

Physical Properties and Inhibition

Optimum activity of the enzyme occurs over the range from pH 5.5 to 9.0 (Dixon and Thurlow, 1924b). Wieland and Rosenfeld (1930) reported the optimum range is pH 8.5 to 9.0. Green (1934) showed that the oxidation-reduction potential is highly negative; E'_o is -0.371 for the hypoxanthine-xanthine system and -0.361 for the xanthine-uric acid system.

Cyanide inhibits xanthine dehydrogenase slowly, irreversibly and eventually completely (Dixon and Keilin, 1936). Both methylene blue reduction and oxygen consumption are inhibited, indicating that the cyanide interacts with the enzyme itself and not with some associated oxygen activator. This inhibition is different from cyanide inhibition of iron-catalyzed reactions which takes place quickly, is reversible and depends on cyanide concentration. Uric acid protects the enzyme from inactivation by cyanide; hypoxanthine protects only in the presence of a hydrogen acceptor. Carbon monoxide, hydrogen sulfide and pyrophosphate do not inhibit.

The purified enzyme is inhibited by traces of copper and it is protected from this inhibition by glycine (Philpot, 1938). Oxidized para-aminophenol is a powerful inhibitor of xanthine dehydrogenase (Bernheim and Bernheim, 1938); 0.008 M solution of free iodine inhibited 88 per cent, and 0.001 M pyrogallol inhibited approximately 44 per cent (Gray and Felsher, 1945). Stadie and Haugaard (1945b) found that the xanthine enzyme of slices and homogenates is rapidly inactivated by oxygen at a pressure of seven atmospheres. These results may indicate that -SH groups are essential for the activity.

URICASE

(Uric Acid Dehydrogenase, Urico-oxidase)

The enzyme which catalyzes the oxidation of uric acid to allantoin was discovered by Schittenhelm (1905). Battelli and Stern (1909) studied this enzyme more extensively and named it "uricase". They found it present in the liver and kidney of various animals. The distribution in most mammals is limited to a few tissues such as the liver, kidney and spleen. It is not found in human tissues and it is of particular interest that man excretes uric acid and not allantoin. The anthropoid apes and the Dalmatian dog excrete little allantoin, although the liver of the latter is rich in uricase.

Florkin and Duchateau-Bosson (1943) made a comprehensive study of the uricolytic enzyme systems in the animal kingdom, including both vertebrates and invertebrates. They found uricase present in coelenterates, echinoderms, supunculids, crustaceans, dipterous insects, lamellibranch and gastropod mollusks, most fishes, batrachians and most mammals. It is absent in platodes, oligochetes, hirudineans, most insects, cyclostomes, snakes and lizards, birds, primates and man.

Uricase is intimately associated with insoluble particles, but is easily prepared in varying degrees of purity from fresh liver and kidney tissues of the cow and pig. A stable crude preparation suitable for experimental study is obtained by grinding the fresh liver and drying it with acetone. Davidson (1938a,b) prepared an extract of pig liver which had about 550 times greater activity than the liver powder. It was soluble at pH 10.0 in borate buffer. It gave a Q_{O2} of 5000. Although the purified material contained 0.15 to 0.2 per cent iron, it probably did not contain hematin compounds because it was colorless. The nitrogen content was 14.4 per cent.

Holmberg (1939) modified the method of Davidson and obtained a preparation which gave a Q_{O2} of 6000. The material was slightly brown in color. It contained 0.025 per cent iron, 13.5 per cent nitrogen and 0.13 per cent Zn ions. It was insoluble in water and only slightly soluble in pH 10.0 buffer.

Oppenheimer and Kunkel (1943) modified the method of Davidson and obtained purified uricase from acetone-dried pig liver by extracting with 0.1 M borate buffer of pH 10.0. The enzyme was precipitated with saturated ammonium sulfate, the precipitate was dissolved and heated to 55°C. to precipitate inactive protein. The enzyme was recovered by precipitation with ammonium sulfate, dissolved in water and dialyzed. The uricase, which precipitated during dialysis, was washed with 0.1 M phosphate buffer of pH 7.4 and dissolved in borate buffer. This procedure was later altered to effect further purification. The purified uricase was made bacteria-free by filtration, and was active for more than two months when kept in the frozen state. Oppenheimer and Kunkel (1943) showed that the injection of this uricase preparation into chickens, in which gout was induced by a high meat diet, resulted in a lowering of plasma uric acid. When the enzyme and meat diet were given simultaneously, the plasma uric acid did not rise to high levels.

Uricase is most active at alkaline pH values, and Keilin and Hartree (1936a) found the optimum to be at pH 9.25. There is little activity below pH 7.0 or above pH 11.0. Holmberg (1939) used oxygen consumption as an index of uricase activity. The uptake was measured at pH 9.0 and 39°C. during the first 15 minutes of action when lithium urate was used as substrate. The potential level of the uric acid-allantoin precursor system is not known.

Specificity and Action

Uric acid is the only compound oxidized by uricase. According to Brunig et al. (1928) and Keilin and Hartree (1936a) it does not catalyze the oxidation of related compounds, such as hypoxanthine and xanthine, or 4,5-glycol, 4,5-dimethyl, 1-,3-,7-,9-methyl, and 16 mono-, di- and trimethyl, or ethyl uric acids. Buchanan, Block and Christian (1945) showed that the uricase in their beef kidney preparation was specific for uric acid. Other substances found in human urine, such as adenine, guanine, xanthine, the methylxanthines, allantoin, various phenols and amino acids, did not interfere with the uric acid determination in which the uricase preparation was used.

According to Keilin and Hartree (1936a) the oxidation catalyzed by uricase is represented by the following equation:

$$\text{Uric acid} + 2H_2O + O_2 \longrightarrow \text{Allantoin} + CO_2 + H_2O_2.$$

The exact mechanism of this oxidation is not known. It is rather certain, however, that the enzyme is not involved in the direct production of allantoin but in the oxidation of uric acid to a precursor of this compound. Schuler and Reindel (1932) obtained evidence which they used as a basis for suggesting that the first oxidation product is oxy-acetylene-diurein-carboxylic acid. Klemperer (1945) showed that only a fraction of one equivalent of carbon dioxide is formed during the oxidation. This was taken to indicate that substances other than allantoin are formed. It was concluded from the amount of nitrogen released on treatment with nitrous acid, that these substances are uroxanic acid and hydroxy-acetylene-diurein-carboxylic acid. Since these compounds and allantoin are stable in the presence of uricase, the primary product of uricase action is believed to be an unstable or precursor compound which decomposes without the intervention of enzymes into the three reaction products. The reaction of the medium and the buffers used determine the relative amounts of the three compounds formed. Hydrolysis, decarboxylation, hydration and dehydrogenation are probably involved in the process. According to Klemperer (1945) the mechanism <u>in vitro</u> is represented by the following equations:

1. $\text{Uric acid} + O_2 + H_2O \longrightarrow \underset{\text{precursor}}{C_5N_4O_4H_4} + H_2O_2$

$$\longrightarrow (a) + H_2O \longrightarrow \text{hydroxyacetylene-diureine-carboxylic acid}$$

2. $\underset{\text{precursor}}{C_5N_4O_4H_4} \longrightarrow (b) + H_2O \longrightarrow \text{allantoin} + CO_2$

$$\text{precursor} \longrightarrow (c) + 2H_2O \longrightarrow \text{uroxanic acid}$$

Keilin and Hartree (1936a) demonstrated that uricase specifically requires oxygen as the hydrogen acceptor. For each molecule of uric acid oxidized, one molecule of oxygen is consumed and one molecule of hydrogen peroxide is produced. That hydrogen peroxide is formed, was shown by the secondary oxidation of para-phenylenediamine in the presence of uricase and uric acid. It has been demonstrated that the velocity of uric acid oxidation depends on the oxygen tension, the rate being twice as great in pure oxygen as in air. There is no other known hydrogen acceptor for the system, and methylene blue and dyes of higher potential are not reduced under anaerobic conditions. This shows that uricase occupies a unique position among the dehydrogenases in that it shows the properties of oxidases. However, Keilin and Hartree classed it as an aerobic dehydrogenase on the basis of its absolute specificity and the production of hydrogen peroxide during the oxidation.

The hydrogen peroxide formed by the action of uricase on uric acid can be used in coupled oxidations. When alcohol is added to the system it undergoes a secondary or coupled oxidation to aldehyde by the hydrogen peroxide formed in the primary oxidation of uric acid to allantoin, and the oxygen uptake is doubled (Keilin and Hartree, 1936b). If a little purified catalase is added to the system, in addition to the alcohol, a cyclic oxidation is established and oxygen is consumed beyond doubling the uptake of the primary reaction.

Keilin and Hartree (1936a) found that several compounds structurally related to uric acid inhibit uricase competitively. The enzyme is almost completely, but reversibly, inhibited by 0.001 M cyanide; activity is restored when the cyanide is removed. Sodium fluoride, hydrogen sulfide, carbon monoxide and ethyl urethane do not inhibit. Cysteine is reported to activate uricase (Scheer and Scheer, 1943).

ALDEHYDE DEHYDROGENASE

(Potato and Liver Aldehyde Oxidases)

Potato and liver tissues contain enzymes in addition to xanthine dehydrogenase which catalyze the oxidation of aldehydes. The following discussion is concerned with these aldehyde dehydrogenases. The potato enzyme is discussed first.

The dehydrogenase of the potato in the presence of aldehydes catalyzes the reduction of nitrate to nitrite (Bach, 1913; Michlin, 1927, 1928). Michlin found that this dehydrogenase does not oxidize hypoxanthine, and Michlin and Severin (1931) showed that it does not catalyze the dismutation of acetaldehyde. It is not inhibited by uric acid (Booth, 1935).

Bernheim (1928a) purified the potato enzyme by fractionation with ammonium sulphate and adsorption on kaolin and showed that it reduces both nitrate and methylene blue in the presence of aldehyde. Bhagvat, as reported in Biological Oxidations by Green (1940), prepared the potato enzyme and studied it systematically. The method of preparation is given and the product is 112 times more active than the crude press juice from which it was made. Franke and Schumann (1942) concentrated aldehyde dehydrogenase from a Na_2HPO_4 extract with ammonium sulfate. The preparation was 125 times more active than the original juice. This preparation was used in an extensive study in which it was concluded that the enzyme is a globulin.

Results obtained by use of these preparations show that the potato enzyme catalyzes the oxidation of both aliphatic and aromatic aldehydes providing nitrate or dyes such as methylene blue are present. Coenzyme is not required. Bhagvat found it to be inactivated on shaking with air in the presence of aldehyde, but destruction did not take place in the absence of substrate. The destruction was shown to be due to the formation of hydrogen peroxide which may account for the failure of the enzyme to oxidize aldehyde in the presence of oxygen. Franke and Schumann (1942) also found that oxygen had a detrimental effect on their purified enzyme.

Bhagvat found that the dehydrogenase is inhibited almost completely by 0.0033 M cyanide, and reversible inactivation resulted from incubation with 0.001 M cyanide. This inhibition with cyanide was confirmed by Franke and Schumann (1942). They also demonstrated complete inhibition with sodium azide, iodoacetate and quinone and partial inhibition was obtained with hydroquinone. Carbon monoxide, sodium fluoride, phenylurethane, phenylurea, chloral hydrate, octyl alcohol, toluene and chloroform did not inhibit.

The Dehydrogenase of Animal Tissues

In the case of the liver enzyme, Lemberg, Wyndham and Henry (1936) found that the livers of the different animals they examined, except those of man and the cow, contained more aldehyde oxidase than xanthine oxidase activity. They concluded that another type of aldehyde oxidase is present. During the study of xanthine dehydrogenase in pig liver, Corran, Dewan, Gordon and Green (1939) found that the ratio of aldehyde activity to xanthine activity decreased markedly during the early stages of purification and then remained constant. They thought this indicated the presence in liver of an enzyme active toward aldehydes but inactive toward xanthine, and that it is separated in the early stages of purification of xanthine dehydrogenase. This led Gordon, Green and Subrahmanyan (1940) to make an intensive study of liver aldehyde dehydrogenase.

Their method of purification consists of the extraction of minced pig liver with a solution of 4 l water and 1840 ml. 97 ethyl alcohol at 48° C., concentration of the enzyme

by precipitation with basic lead acetate, elimination of the lead as lead phosphate, and purification of the enzyme by a series of ammoniacal ammonium sulfate precipitations. During the course of this purification the hemoglobin, xanthine dehydrogenase flavoprotein, a $Fe(OH)_3$-protein complex, and the major part of the catalase of the original extract are eliminated. The final preparation of the enzyme, which is probably 50 per cent pure, is associated with a colored compound other than flavin. The best preparations contained 0.17 per cent flavinphosphate.

The flavin group can be split from the enzyme by boiling, by acidification to pH 4.0 or by prolonged dialysis. This group was separated from the enzyme and purified and its identity with yeast flavin adenine dinucleotide was established by use of the D-amino acid oxidase test.

In the presence of acetaldehyde the enzyme undergoes a cycle of reduction by the substrate and oxidation by molecular oxygen. Absorption spectra changes showed that it is the flavin group of the molecule which is reversibly reduced and oxidized. This indicates that this group is a part of the enzyme molecule. In the presence of acetaldehyde the enzyme is oxidized and reduced about 550 times per minute.

Determination and Mechanism of Action

Liver aldehyde dehydrogenase catalyzes the oxidation of aldehydes in the presence of molecular oxygen and certain dyes such as methylene blue. Methods for determining the activity are based on these reactions. A Q_{MB} of 2700 was obtained by using the methylene blue test with acetaldehyde as the substrate. The reaction with oxygen is rapid and obviously it must be the physiological hydrogen acceptor. Nitrate and cytochrome c are reduced at a slow rate.

The liver enzyme catalyzes the oxidation of various aliphatic and aromatic aldehydes, such as acetaldehyde, propionaldehyde, butyraldehyde, crotonaldehyde and benzaldehyde. Ketones, acids and alcohols are not attacked. The purified enzyme does not catalyze the oxidation of hypoxanthine or reduced DPN.

The aldehydes are oxidized to the corresponding acids. One atom of oxygen is consumed per molecule of aldehyde oxidized. That the product of the reaction is acetic acid when acetaldehyde is used as substrate, was demonstrated by use of the distillation method of Dyer for the identification of volatile organic acids.

The half-maximum rate of oxidation was obtained with 0.007 M crotonaldehyde. Maximum velocity is shown at pH 7.0, although the enzyme is active over the range of pH 5.0 to pH 11.0.

It should be pointed out that both the liver aldehyde and xanthine dehydrogenases oxidize aldehydes at practically the same rate, and that this activity is irreversibly destroyed by incubation with cyanide and also by desiccation. On the other hand the two enzymes have different solubilities in ammonium sulfate solution. The liver enzyme is irreversibly destroyed by dialysis, whereas xanthine dehydrogenase is reversibly and only partially inactivated by prolonged dialysis. The cyclical oxidation and reduction of the flavin group of liver dehydrogenase is easily demonstrated. This is not true for the xanthine enzyme. On the basis of these different properties, liver aldehyde dehydrogenase is considered to be distinct from xanthine dehydrogenase.

D-AMINO ACID, L-AMINO ACID, GLYCINE, MONOAMINE and DIAMINE DEHYDROGENASES are discussed in the chapter on NITROGEN METABOLIZING ENZYMES.

General Summary of Information on the Aerobic Dehydrogenases

The dehydrogenases of the aerobic group have been purified to a considerable degree. Xanthine, D-amino acid, L-amino acid, glycine and liver aldehyde dehydrogenases are flavoproteins. Flavin adenine dinucleotide is the prosthetic group of these enzymes, with exception of the L-amino acid enzyme which has flavin mononucleotide for its prosthetic group. Uricase and the two amine dehydrogenases appear not to be flavoproteins.

The aerobic dehydrogenases are group-specific, with the exception of uricase and glycine dehydrogenase. In addition xanthine dehydrogenase activates both purines, and aliphatic and aromatic aldehydes. L-Amino acid dehydrogenase oxidizes both L-amino and L-hydroxy acids.

The action of glycine, D-amino acid, L-amino acid, and amine and diamine dehydrogenases is explained by essentially the same mechanism. Two hydrogens are removed to give the imine compounds which, in the presence of water, spontaneously form the corresponding keto acids and aldehydes.

The aerobic dehydrogenases react directly with molecular oxygen to form hydrogen peroxide and, with the exception of uricase and the amine dehydrogenases, they reduce methylene blue. Uricase is highly specific for oxygen. Methods for the quantitative determination of the activity of these enzymes are usually based on oxygen consumption or methylene blue reduction. The diamine and xanthine dehydrogenases, uricase and aldehyde dehydrogenase of potato are inhibited by cyanide. The Michaelis constant and the pH of optimum activity for the aerobic dehydrogenases are given in Table III.

TABLE III

Physical Constants of Aerobic Dehydrogenases

Dehydrogenase	pH of Optimum Activity	Michaelis Constant K_m (in molarity of substrate)
Xanthine	8.9-9.0 Wieland and Rosenfeld (1930)	$< 1.5 \times 10^{-4}$ (hypoxanthine)
	5.5-9.0 Dixon and Thurlow (1925b)	<0.06 (acetaldehyde)
D-Amino acid	8.6 Krebs (1939)	0.005 (DL-alanine)
L-Amino acid	10.0 Blanchard et al. (1944)	0.009 (L-N-methylleucine)
L-Hydroxy acid	8.0 Blanchard et al. (1946)	0.004 (L-lactate)
Glycine	8.3 Ratner et al. (1944)	0.04 (glycine)
Uric acid (uricase)	9.25 Keilin and Hartree (1936a)	
Aldehyde (animal)	7.0 Gordon et al. (1940)	0.007 (crotonaldehyde)
Amine	7.3 Bhagvat et al. (1939)*	0.0018 (tyramine)
Diamine	6.8-7.6 Zeller (1942)*	<0.0005 (histamine)

*The pH values given for amine and diamine dehydrogenases are those used for the quantitative estimation of these enzymes.

ANAEROBIC DEHYDROGENASES (Cytochrome-linked)

SUCCINIC DEHYDROGENASE

The presence of succinic dehydrogenase in tissues was first discovered by Battelli and Stern (1910a) through the observation that when succinate was added to certain tissues the oxygen uptake was markedly increased and malic acid was produced. Fumaric acid was found later to be the primary reaction product. In the presence of fumarase it forms an equilibrium with malic acid.

More recent work has shown that the catalytic dehydrogenation of succinate is the result of the action of succinic dehydrogenase, cytochrome c and cytochrome oxidase. The components of this reaction are often referred to as the "succinoxidase system".

Succinic dehydrogenase is widely distributed in nature. According to Elliott (1941) it is found in bacteria, molds, insects and most of the tissues of vertebrates that have been tested. It varies greatly in activity from tissue to tissue, as shown by the results of various workers. Elliott and Greig (1938) studied the distribution of this enzyme in animal tissues. Schneider and Potter (1943a) determined the activity in heart, kidney, liver, brain, skeletal muscle, spleen, lung and Jensen sarcoma of the rat. Its activity in endocrine tissues was reported by McShan and Meyer (1946a). High activity is found in heart, kidney and liver; moderate activity in brain, skeletal muscle, adrenal and functional lutein tissue; and low activity in spleen, lung, many cancerous tissues, thyroid, pancreas, early embryonic tissue, uterus, thymus, nonfunctional lutein tissue, testis, prostate and seminal vesicle.

Several reports have recently appeared on the correlation of changes in succinic dehydrogenase activity of various tissues with changes in function of these tissues. The activity in the liver of rat embryos increases rapidly during late embryonic and early post-natal life and reaches the adult level within 15 days after birth (Potter, Schneider and Liebl, 1945). The activity in liver tumors is about one-fourth that of normal liver (Schneider and Potter, 1943b), it is much lower in rat hepatoma than in normal liver (Schneider, 1946b), it increases in developing chick embryos from 8 to 21 days (Albaum, Novikoff and Ogur, 1946), and Meyer et al. (1947) found that the activity in functional corpora lutea of rat ovaries during pregnancy is two and one-half times greater than in non functional diestrous corpora. Results of studies of this kind may help provide the basis for an insight into the mechanism of certain of these specialized physiological processes.

Preparation and Purification

Succinic dehydrogenase is attached to insoluble cellular constituents and consequently it is not readily purified. Ohlsson (1921) used weakly alkaline phosphate solution to obtain an active opalescent extract from washed minced horse muscle. Ogston and Green (1935) partially purified the enzyme by grinding minced cow or sheep heart muscle with sand and straining through cheese-cloth. The enzyme was recovered from the extract by precipitation at pH 4.6. They also extracted minced liver with Ringer solution and precipitated the enzyme with ammonium sulfate. A combination of the above procedures was used by Weil-Malherbe (1937c) to prepare a partially-purified, dry, stable preparation. These preparations contained cytochrome oxidase, but much of the cytochrome c was removed during the process of preparation, particularly when liver was used. As a result the oxygen uptake was markedly increased by adding cytochrome c to the liver preparation in the presence of succinate.

Stotz and Hastings (1937) used essentially the method reported by Borsook and Schott (1931) to prepare the dehydrogenase free of fumarase. Fat-free beef heart muscle was extracted with water at 45° C., the liquid was separated, the residue was ground and the enzyme was extracted with 0.067 M K_2HPO_4.

Hogeboom (1946) reported that about 70 per cent of the succinoxidase (succinic dehydrogenase plus cytochrome oxidase) content of the cytoplasmic extract of rat liver had been recovered in a large granule fraction consisting mainly of mitochondria by sedimentation at 2700 times gravity. This was confirmed by Schneider (1946a), Hogeboom, Claude and Hotchkiss (1946) and by Hogeboom, Schneider and Pallade (1947).

Hogeboom (1946) prepared succinic dehydrogenase from the mitochondrial fraction of guinea pig liver. After washing the granules once with 0.85 per cent NaCl containing 0.025 M $NaHCO_3$, they were treated with cold acetone, dried quickly *in vacuo*, and extracted for 18 hours at 0° C. with 0.01 M $NaHCO_3$. A clear yellow solution of the dehydrogenase was obtained by centrifuging at 18,000 times gravity. Approximately 10 per cent of the enzyme activity was recovered from the granules by this procedure. The soluble dehydrogenase was precipitated between 20 and 50 per cent saturation with ammonium sulfate. When this fraction was dissolved in 0.01 M $NaHCO_3$, 90 per cent of the activity remained after four days at 0° C. It was not affected by dialysis against dilute bicarbonate. The preparation was free of cytochrome oxidase.

Determination and Specificity

The succinic dehydrogenase activity of various kinds of tissues and preparations has been studied by determining the rate of reduction of methylene blue and by the measurement of oxygen uptake in the presence of the cytochrome system.

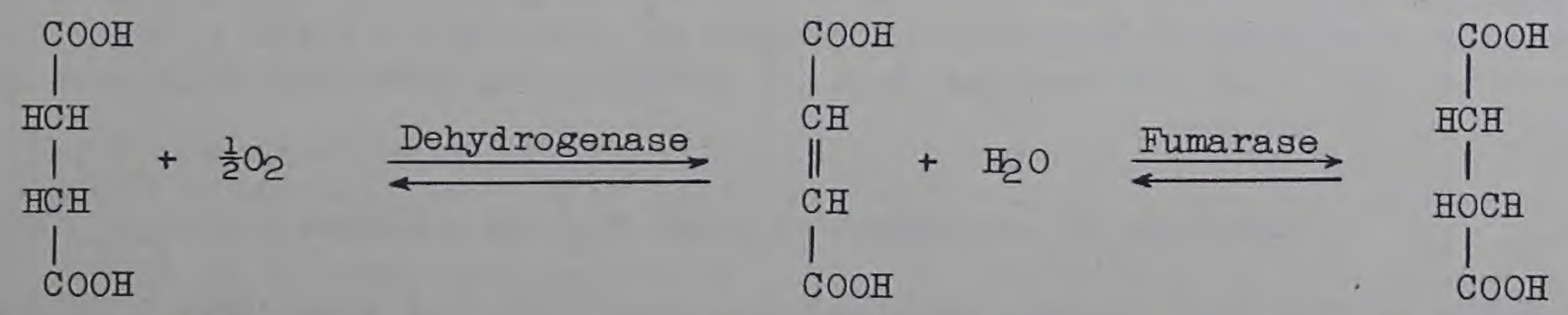
A method of determining the activity of tissue homogenates was reported by Schneider and Potter (1943a). It is necessary to add cytochrome c, and Al and Ca ions to the homogenate to obtain maximum activity (Potter and Schneider, 1942). That traces of Al ions increase the rate of oxygen consumption by the succinoxidase system was first shown by Horecker, Stotz and Hogness (1939). Elliott and Greig (1938) showed that when the cytochrome concentration is low, addition of cytochrome c accelerates the oxidation of succinate. According to Axelrod, Swingle and Elvehjem (1941), and Swingle, Axelrod and Elvehjem (1942) Ca ions activate crude succinic dehydrogenase preparations by increasing the rate of destruction of DPN. When DPN is not destroyed, the malic dehydrogenase present oxidizes malate to oxalacetate and the latter inhibits succinic dehydrogenase. In order to overcome the dilution effect and maintain activity, Schneider and Potter (1943a) varied the cytochrome c and Al and Ca ions to find the proper concentrations for optimum activity. In this way the enzyme is purposely made the limiting factor in the system and the oxygen uptake is a measure of the maximum activity of the dehydrogenase.

The composition of the reaction mixture found to give optimum activity consists of 1 ml of 0.1 M phosphate buffer of pH 7.4, 0.4 ml of 10^{-4} M cytochrome c, 0.3 ml of 0.5 M sodium succinate, 0.3 ml each of 0.004 M $CaCl_2$ and 0.004 M $AlCl_3$, plus the desired amount of homogenate, and sufficient water to make a total volume of 3 ml. The oxygen consumption of the fortified system is measured manometrically in Warburg vessels.

Specificity studies by Thunberg (1933) showed that succinic dehydrogenase activates succinic acid, and also methyl succinic slightly. Franke and Siewardt (1944) found that ethyl succinic acid is slowly attacked.

Mechanism of Action

Einbeck (1914, 1919) showed that muscle causes the oxidation of succinate to fumarate with the uptake of one atom of oxygen per molecule of succinate oxidized and that most tissues contain an enzyme, fumarase, which converts fumarate to malate. When equilibrium is established there are three molecules of malate present to one of fumarate. This overall reaction is represented as follows:



Succinic acid — Fumaric acid — Malic acid

It was shown by Thunberg (1918, 1923) and others that in the presence of washed muscle tissue, succinate rapidly reduced methylene blue. Cyanide did not inhibit this reduction. Szent-Gyorgyi (1924), and Fleisch (1924) showed that oxygen uptake, but not methylene blue reduction, is inhibited by cyanide. It was concluded from these results that, in order for oxygen consumption to occur, succinate and oxygen must be activated by two different mechanisms. Later work by Stotz and Hastings (1937) confirmed this conclusion. Keilin (1929) clarified the mechanisms involved by showing that succinic dehydrogenase in the presence of succinate reduces the components of cytochrome and the reduced cytochrome is reoxidized by the cyanide sensitive cytochrome system. When an autoxidizable dye such as methylene blue is reduced by the dehydrogenase in the presence of succinate, the dye is reoxidized directly by oxygen. This reaction is insensitive to cyanide. When cytochrome c is likewise reduced, cytochrome oxidase in the presence of oxygen cata-

lyzes its reoxidation. This reaction is inhibited by cyanide and the inhibition is mediated through the cytochrome oxidase.

Ball (1938) showed that when succinate is added to a cytochrome suspension, cytochromes a, b and c are reduced. Euler and Hellstrom (1939) found that cytochromes a and b are reduced by the dehydrogenase, but it is not clear what part they play in the functioning of the system. According to Potter and Lockhart (1939) cytochrome b may be required as an intermediate carrier for the catalytic oxidation of succinate. Potter (1941) studied the mechanism of hydrogen transport by the succinoxidase system and showed methylene blue to be much less effective than cytochrome c as a hydrogen carrier. Andersson (1934) showed that this system does not require pyridine nucleotide. Dewan and Green (1937) found, however, that the dehydrogenase in the presence of fumarate can oxidize reduced DPN and in the process fumarate is reduced to succinate.

The effect of succinic dehydrogenase on α, α'-dideuterosuccinate in the absence of acceptors was studied by Weinmann, Morehouse and Winzler (1947). They found that there was a definite exchange between the deuterium of succinate and the hydrogen of the water molecules both in the absence and presence of the enzyme. In the presence of the enzyme, however, the rate of exchange was increased in proportion to the increase in the rate of succinate oxidation. Succinic dehydrogenase, therefore, catalyzes (1) a transfer of hydrogen from succinate to an appropriate acceptor, and (2) an exchange between the α -hydrogen of succinate and the hydrogen ions of the medium. The viewpoint is favored that the substrate hydrogen dissociates into proton and electron directly from an enzyme-substrate complex. This is in accord with the theory of Potter and DuBois (1943) in which the enzyme function is represented as an oscillation between the normal thiol form and its free radical with electrons passing to the prosthetic group of the enzyme and hydrogen ions passing into the medium.

Quastel and Whetham (1924) demonstrated that succinic dehydrogenase catalyzes a truly reversible reaction. Their proof consists of the fact that washed bacteria catalyze the oxidation of succinate in the presence of methylene blue and the reduction of fumarate to succinate with the resulting oxidation of leuco-methylene blue. Thunberg (1925) confirmed these findings using the dehydrogenase of muscle and also used his data to calculate the oxidation-reduction potential of the succinate-fumarate system. A more complete study was made by Lehmann (1930). Borsook and Schott (1931) showed that the enzyme behaves as a perfect catalyst and concluded that it probably operates in the same manner *in vivo* as *in vitro*. The E_0' for the system at 37° C. and pH 7.0 is 0.0 V.

Optimum activity of the enzyme in muscle and bacteria was found by Ohlsson (1921) and Cook and Alcock (1931) to be at approximately pH 9.0. Laki (1938) found the optimum reduction of methylene blue at pH 8.8. The pH of optimum activity varies depending on the dye used. With muscle maximum consumption of oxygen is reported to occur at pH 7.6. In the presence of methylene blue the maximum rate of oxidation is obtained with 0.01 M and half-maximum with 0.001 M succinate.

Linkage of the Dehydrogenase with Cytochrome c

The results of Hopkins, Lutwak-Mann and Morgan (1939) indicate that a factor in addition to the known coenzymes and carriers is necessary for the reduction of cytochrome c by succinic dehydrogenase. Horecker, Stotz and Hogness (1939) showed that traces of Al and Cr ions increase the rate of oxygen uptake by the succinoxidase system, but these ions do not affect the activity of the cytochrome system or the rate of dye reduction. In the light of this it was thought that Al ions might be the additional factor Hopkins et al. (1939) found necessary for the linkage of succinic dehydrogenase to the cytochrome system.

Stern and Melnick (1939) ultracentrifuged the heart muscle preparation of Keilin and Hartree (1938) and reported the separation of it into two aerobically inactive fractions which, when recombined, formed a complete system because under aerobic conditions succinate was oxidized. Keilin and Hartree (1940) questioned this result since they could wash their preparation several times without great loss of activity.

When Keilin and Hartree (1940) treated the heart muscle preparation with acid or pancreatin, fractions were obtained in which both the dehydrogenase and cytochrome oxidase were present, but the system was incomplete because these fractions did not catalyze the aerobic oxidation of succinate. It was suggested, on the basis of these results, that succinic dehydrogenase reduces cytochrome c through some labile intermediate compound, such as cytochrome b or some unknown component of the system, or that the dehydrogenase in preparations of this kind has lost its accessibility to cytochrome c but not to the more diffusible molecules of methylene blue.

Straub (1942a) demonstrated that an unknown factor is necessary for the coupling between succinic dehydrogenase and cytochrome c. This unknown factor, designated as the SC factor, was obtained from pig heart muscle. It is inactive as an intermediate for malic acid oxidation since it does not mediate the reaction between diaphorase and cytochrome c. Stoppani (1947) obtained a soluble factor from ox and rabbit liver which links succinic dehydrogenase to the cytochrome system. It cannot be replaced by diaphorase, malic dehydrogenase, fumarase, catalase, flavin dinucleotide, riboflavin, cysteine, glutathione and Ca or Al ions in concentrations of 10^{-5} M. These results show that an unknown factor is required for the reaction of succinic dehydrogenase with the cytochrome system, but further work is necessary to elucidate the exact mechanism.

Inhibition

Quastel and Wheatley (1931) showed that malonate inhibits succinic dehydrogenase; it also prevents the formation of succinate from fumarate and oxalacetate. Krebs (1943) emphasized the importance of malonate inhibition of succinic dehydrogenase as supporting evidence for the citric acid cycle hypothesis, since it was shown that succinate can be formed from fumarate in the presence of malonate providing oxygen is present. Das (1937b) reported that oxalacetate is a strong inhibitor of this dehydrogenase. Other workers have shown that compounds, such as fumarate, oxalate, methyl succinate, glutaric, adipic and suberic acids inhibit to a lesser degree. These compounds are structurally related to succinate and have an affinity for the dehydrogenase which results in their adsorption on the enzyme. This prevents the dehydrogenase from activating the substrate, succinate. This is competitive inhibition since the inhibitor competes with the substrate for the enzyme.

The split products of paradimethyl-aminoazobenzene inhibit succinic dehydrogenase and other -SH containing enzymes such as urease (Potter, 1942). Ames and Elvehjem (1944, 1945) studied the inhibition of the enzyme by cysteine and cystine, with particular emphasis on the mechanism of the inhibition and the conditions suitable for inhibition to take place. Synthetic estrogens, such as diethylstilbestrol and benzestrol, are effective inhibitors for the succinoxidase system, but this inhibition is mediated through the cytochrome oxidase of the system (McShan and Meyer, 1946b).

Adler, Euler and Skarzynski (1943) and Zittle (1946) showed that yeast adenylic acid, adenosine, nucleic acids and their hydrolytic products inhibit succinic dehydrogenase. This inhibition increases with time and it is apparently noncompetitive. Desoxyribose nucleic acid, which is intimately associated with the cell nucleus and chromosome structure, is much more effective than is the cytoplasmic ribose nucleic acid. The mechanism and its physiological significance are not clear from present results. According to Greenstein and Chalkley (1945) results of this kind are probably the first direct chemical evidence that a natural nuclear constituent can directly affect the enzymatic systems of the cell.

Inactivation and Essential Groups

The succinic dehydrogenase of washed tissue was inactivated by Hopkins et al. (1938) by anaerobic incubation with oxidized glutathione. This inactivation was reversed and the activity restored by incubation with reduced glutathione. On the basis of this evidence it was concluded that the enzyme is dependent on intact -SH groups for its activity. Further evidence to support this concept is the fact that the enzyme is inactivated by Cu ions, maleic and iodoacetic acids and alloxan, all of which are known to react with -SH

groups. Malonic, succinic and fumaric acids protect the dehydrogenase from oxidized glutathione. Euler and Hellstrom (1938) conclude, on the basis of results obtained with oxidizing and reducing agents, that the activity depends on -SH groups.

Potter and DuBois (1943) extended the study of the mechanism of succinic dehydrogenase action by use of inhibitors. They found that the enzyme is inhibited by a large number of different kinds of compounds, such as quinones, -SH reagents, -SH compounds, Cu and Zn ions, selenite and arsenite, whose only obvious common property is their ability to react with -SH groups. Inhibition by malonate and these -SH reactants was shown to be mutually exclusive. On the basis of these data it was concluded that the -SH groups of the enzyme which are essential for its activity are located between the carboxyl affinity points. Consequently when malonate is adsorbed on the enzyme, it not only inhibits but also protects the -SH groups from reagents that would otherwise react with them and cause inactivation. On the basis of this structure, Potter and DuBois (1943) suggested that the enzyme functions, not by a thiol-disulfide equilibrium, but by oscillating between the EnSH and EnS· forms. This concept may provide the explanation for the role of thiol groups in the mechanism of hydrogen transfer.

Gordon and Quastel (1947) reported that arsenoxide (RAsO) compounds, such as meta-amino-para-hydroxy phenylarsenoxide, combine reversibly with -SH groups and are therefore inhibitors of enzymes, such as succinic dehydrogenase, whose activities are dependent on -SH groups. Stadie and Haugaard (1945a) showed that succinic dehydrogenase is inactivated after prolonged exposure to eight atmospheres of oxygen. This inactivation is believed to be due to the oxidation of -SH groups, since the enzyme is protected in the presence of malonate or high concentrations of succinate. Haugaard (1946) reported a high degree of correlation between susceptibility of enzymes to the action of oxygen and the presence of essential -SH groups.

α-GLYCEROPHOSPHATE DEHYDROGENASE (Cytochrome)

Meyerhof (1919) showed that glycerophosphate is oxidized on mixing with washed liver or muscle. Linkage with the phosphate group was found to be necessary because the oxidation did not take place when glycerol was used as substrate. Several other workers have studied the properties of this enzyme. The evidence indicates that there are two glycerophosphate dehydrogenases. The one discussed here catalyzes the direct reduction of cytochrome. Euler, Adler and Gunther (1937a) showed that the other glycerophosphate dehydrogenase is active only in the presence of DPN and it is discussed elsewhere.

The cytochrome-linked dehydrogenase resembles succinic dehydrogenase in many respects. They are found in many tissues and both are associated with insoluble material. The stability of the two enzymes is similar. They are not identical, however, since Alwall (1930) showed that the rates of oxidation of their substrates varies widely for different tissues. For example, the glycerophosphate enzyme is more active in skeletal than in heart muscle, whereas the reverse holds for the succinic enzyme. There is an additive effect on the rate of methylene blue reduction in the presence of appropriate concentrations of the two substrates (Ahlgren, 1925). Glycerophosphate dehydrogenase does not depend on -SH groups for its activity as has been shown to be the case for the succinic enzyme.

Alwall (1930) found glycerophosphate dehydrogenase in all animal tissue examined. High activity is found in brain, liver and kidney. It is also found in bacteria, yeasts and certain seeds and pollen as indicated by Elliott (1941).

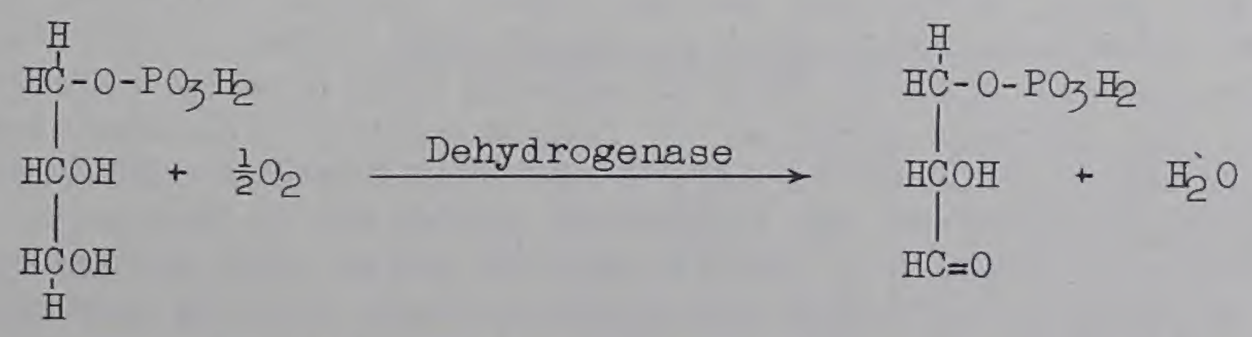
Green (1936a) prepared the cytochrome-linked dehydrogenase and used the preparation in extensive studies. Washed, minced rabbit muscle was ground with sand and strained through cheesecloth. The extract was adjusted to pH 4.6 with acetate buffer. The precipitate which contained the enzyme was recovered and suspended in pH 7.2 phosphate buffer for use. It was stable when dried *in vacuo*, but became unstable after being suspended in buffer for a few days.

The activity of this dehydrogenase is determined by manometrically measuring the consumption of oxygen in Warburg manometers in the presence of substrate and methylene blue or cytochrome c. Under anaerobic conditions the Thunberg method can be used.

The dehydrogenase is specific for the natural L-α-glycerophosphate and the theoretical oxygen uptake was obtained with this compound. When a preparation containing both optical isomers was used, the uptake was only half the theoretical for oxidation to triosephosphate. The oxidation of β-glycerophosphate, glycerol and 2- and 3-phosphoglycerates is not catalyzed.

Mechanism of Action

The enzyme contained in the preparation of Green (1936a) catalyzed the uptake of little oxygen in the presence of α-glycerophosphate, but the uptake was markedly increased on addition of cytochrome c. Adrenaline, ascorbic acid, lactoflavin, glutathione, yellow enzyme, DPN and TPN did not cause an increase in oxygen consumption. This shows that they cannot replace cytochrome c in the system. Since cytochrome oxidase was found to be present in the preparation, the complete system appears to consist of α-glycerophosphate, dehydrogenase, cytochrome c, cytochrome oxidase and oxygen. The reaction is represented as follows:



α-Glycerophosphate 3-phosphoglyceraldehyde

The indications are that glyceraldehydephosphate is the first oxidation product and it breaks down to give methylglyoxal and phosphoric acid, but the formation of these products lags behind the oxygen uptake. The cyanhydrin of the reaction product was isolated as the barium salt and identified from its hydrolytic products as glyceraldehydephosphate. Methylglyoxal was also isolated as the dinitrophenylosazone. The possibility that dihydroxyacetonephosphate is also formed is not excluded.

Glycerophosphate dehydrogenase reduces methylene blue under anaerobic conditions and in the presence of oxygen the dye acts as carrier. The affinity for methylene blue is low, and cytochrome c causes rapid uptake of oxygen at much lower concentrations than are required with methylene blue. The substrate concentration for half-maximum activity depends on the conditions under which the system acts; it has been reported as 0.001 M for methylene blue reduction and 0.01 M for oxygen consumption.

The activity is optimum between pH 8.0 and 10.0 as indicated by oxygen uptake. The oxidation-reduction potential of the system is close to -0.25 V at pH 7.0. Oxidation appears to be the only physiological function of this cytochrome-reducing glycerophosphate dehydrogenase. It is not concerned with glycolytic processes (Green, 1936a).

According to Weil-Malherbe (1937a) 0.01 M cyanide in the presence of methylene blue does not inhibit α-glycerophosphate dehydrogenase, but instead an increase in oxygen consumption actually takes place. This is probably due to the inhibition of catalase, so that hydrogen peroxide accumulates instead of being broken down to oxygen and water. Green (1936a) showed that narcotics such as urethane and octyl alcohol inhibit, but cyanide, azide, fluoride and iodoacetate have little effect, except cyanide inhibits the system in the presence of cytochrome c. Compounds such as 2- and 3-phosphoglycerate which are not activated by the enzyme cause inhibition which is probably competitive in nature. β-Glycerophosphate does not exert any effect.

LACTIC DEHYDROGENASE (Yeast)

Lactic dehydrogenase of yeast is cytochrome-linked and does not require coenzyme as contrasted to the lactate-activating enzyme of animal tissues and certain bacteria which requires DPN. According to Meyerhof and Lohmann (1926), both forms of lactate are oxidized by intact yeast at the same rate. This suggests the presence of two lactate-activating enzymes, unless a racemizing agent is present in living yeast. The results given below, however, show that the cytochrome-linked dehydrogenase which oxidizes L-lactic acid can be prepared from yeast in purified form.

The yeast enzyme is the only one of the cytochrome-linked group of dehydrogenases that is readily obtained in the soluble form. Bernheim (1928b) extracted acetone-dried yeast with Na_2HPO_4 solution and obtained on dialysis an active, cloudy solution of the enzyme. Ogston and Green (1935) prepared a soluble extract of the enzyme. Gurchot and Lowman (1936) made a soluble preparation by eliminating protein from macerated yeast by shaking with ether, followed by precipitation with ammonium sulfate. The enzyme contained in the precipitate was stable in the dry form.

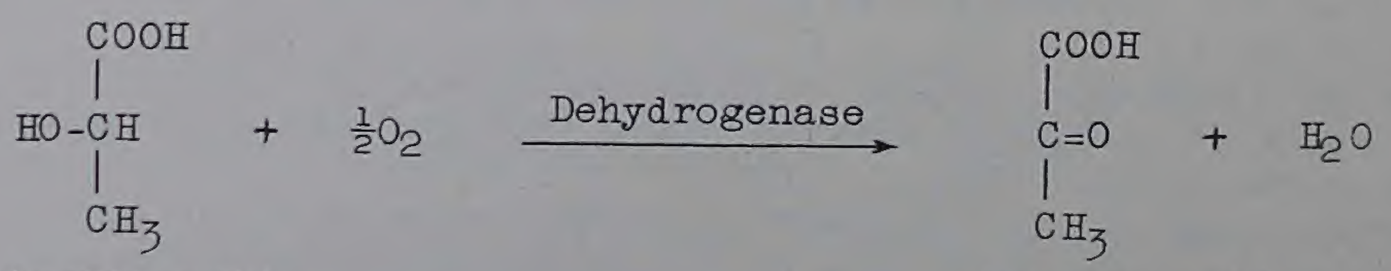
Bach, Dixon and Zerfas (1942) obtained a solution of lactic dehydrogenase from yeast which was 5000 times as active as the preparation of Bernheim. The enzyme was unstable in the absence of lactate. Bach, Dixon and Zerfas (1946) further purified yeast lactic dehydrogenase and obtained solutions which were at least 1000 times as active as those previously known. The enzyme was removed from an aqueous solution by adsorption on calcium phosphate gel, eluted, and further purified by fractionation with ammonium sulfate. They indicate that the dehydrogenase is still impure but that further purification is difficult, due to the increasing instability of the enzyme as purification proceeds. The concentrated enzyme fraction was found to contain a new cytochrome which they designated as b_2. It is soluble in water and apparently is a hemochromogen protein closely associated with cytochrome b. Bach et al. (1946) indicate that cytochrome b_2 is either the dehydrogenase itself, or an essential intermediary carrier between lactate and methylene blue. Other evidence was obtained which indicates that an additional factor is necessary to enable the system to react with cytochrome c.

The activity of lactic dehydrogenase of yeast is estimated quantitatively by determining the time required for the enzyme to decolorize methylene blue in the presence of lactate and the absence of oxygen, or by manometrically measuring the oxygen uptake in the presence of the cytochrome system.

Specificity and Mechanism of Action

Bernheim (1928b) showed that his solution of the yeast enzyme catalyzes the oxidation of lactate to pyruvate. α-Hydroxybutyrate was oxidized less rapidly. Malate, β-hydroxybutyrate, fumarate, citrate, pyruvate and certain other compounds were not oxidized. This enzyme activates only α-hydroxy acids with a levo configuration such as L-lactate. In the presence of pyruvate the enzyme catalyzes the oxidation of reduced DPN (Warburg and Christian, 1936).

The oxidation is represented as follows:



L-Lactic acid — Pyruvic acid

The preparations of Bernheim (1928b) and Gurchot and Lowman (1936) reduced methylene blue in the presence of lactate, but did not take up oxygen unless a dye was added as a carrier. When Ogston and Green (1935) added cytochrome c to their preparation, the oxygen uptake was increased and it was markedly increased when a suspension of heart muscle cyto-

chrome oxidase was added with the cytochrome c. This increased oxygen uptake was inhibited by cyanide, but the reduction of methylene blue was not inhibited. These results show that lactic dehydrogenase of yeast is linked to the cytochrome system. The fact that the purified enzyme of Bach, Dixon and Zerfas (1942) reduced methylene blue but not cytochrome c, suggests that an additional factor is required to link the dehydrogenase to the cytochrome. This was found to be the case for succinic dehydrogenase, as has been indicated elsewhere.

Boyland and Boyland (1934) and Adler and Michaelis (1935) showed that yeast dehydrogenase is not activated by adding DPN and TPN to the system. The fact that dialysis was used by Bernheim (1928b) in making his preparation also suggests that coenzyme is not required.

Boyland and Boyland (1934) showed that when the enzyme is in solution the pH of optimum activity is 6.2 and that when it is used in suspension, as in the case of washed zymin, the optimum is pH 5.6. Bach et al. (1946) reported the optimum activity of their purified enzyme at pH 5.2.

Adler and Michaelis (1935) found that lactic dehydrogenase is not inhibited by fluoride, but arsenite inhibits competitively. It is strongly inhibited by pyruvate, oxalate and glycerate (Bernheim, 1928b).

CHOLINE DEHYDROGENASE

Bernheim and Bernheim (1933) showed that acetylcholine is oxidized in the presence of a suspension of rat liver. This was indicated by an increase in oxygen uptake which was proportional to the amount of acetylcholine added. The acetylcholine iodide used in these experiments was destroyed, and since acetate and potassium iodide caused no increase in oxygen consumption, it was concluded that the choline was oxidized. Fluoride and physostigmine, which are known to inhibit liver esterases such as choline esterase, completely inhibited oxygen uptake. This indicates that the acetylcholine is broken down before the choline is oxidized. Trowell (1935), using rat liver slices, also showed that choline causes oxygen uptake. These results were confirmed by Mann and Quastel (1937).

Trowell (1935), Mann and Quastel (1937) and Bernheim and Bernheim (1933, 1938) showed that choline dehydrogenase occurs in cat and rat liver and kidney, but that it is not present in rat brain, blood, spleen, heart or skeletal muscle. Others have shown it to be absent from the nuclei of liver and certain cancerous tissue cells.

Bernheim and Bernheim (1938) obtained a partially purified but insoluble preparation of choline dehydrogenase from chopped rat liver by grinding with sand and pH 7.8 phosphate buffer. The suspension was strained and dialyzed against distilled water. The resulting precipitate was washed with water and suspended in phosphate buffer in which form the enzyme was stable for several days. This preparation catalyzed the oxidation of succinate, proline and tyramine. The dehydrogenases which oxidize these compounds, however, are found in preparations that are unable to oxidize choline and are, therefore, distinct from choline dehydrogenase.

The Bernheims also showed that an extract can be obtained from acetone-treated liver which will oxidize alcohol but not choline. This indicates that alcohol and choline dehydrogenases are not identical, although both these compounds are oxidized at the primary alcohol group. These compounds are also oxidized by untreated liver suspension, but the oxygen uptake in their presence is additive, which indicates the presence of different enzymes.

Reaction Products and Mechanism of Oxidation

Bernheim and Bernheim (1933) found that one and a half atoms of oxygen were consumed for each molecule of acetylcholine oxidized. Mann and Quastel (1937) obtained large increases in oxygen uptake with extract of liver and also with liver slices. They showed that the increase in respiration is accompanied by the disappearance of choline, indicating that choline is oxidized. They estimated the choline by the reineckate and periodide

methods. Betaine aldehyde was also isolated from a mixture of the reineckates and identified; it is the primary product formed when choline is oxidized by the dehydrogenase.

The preparation of Bernheim and Bernheim (1938) used little oxygen or methylene blue when tested alone, but in the presence of choline and at pH 6.7 one atom of oxygen was consumed for each molecule of substrate oxidized. At pH 7.8 two atoms of oxygen were consumed. Evidence from chemical tests indicates that betaine aldehyde is formed at pH 6.7 and betaine at pH 7.8. Further support for this conclusion is the fact that synthetic betaine aldehyde was oxidized only at pH 7.8. At pH 6.7 choline was oxidized only to betaine aldehyde, but at pH 7.8 it was oxidized completely to betaine. The overall oxidation of choline to betaine aldehyde is represented as follows:

$$\underset{\text{Choline}}{(CH_3)_3N\text{-}CH_2\text{-}CH_2OH} + \tfrac{1}{2}O_2 \xrightarrow{\text{Dehydrogenase}} \underset{\text{Betaine aldehyde}}{(CH_3)_3\overset{+}{N}\text{-}CH_2\text{-}CHO} + H_2O.$$

Mann, Woodward and Quastel (1938) studied the oxidation of both choline and arsenocholine chlorides by choline dehydrogenase obtained from rat liver. They stopped the oxidation of both compounds at the aldehyde stage by use of semicarbazide as a fixative. Under these conditions the oxygen uptake was approximately that required for the formation of the aldehydes in that one atom of oxygen was consumed for each molecule of substrate oxidized. In the absence of semicarbazide the uptake was almost equivalent to one molecule of oxygen to one molecule of substrate oxidized, and in this case the reaction product was betaine. It is clear that these results are in agreement with those of Bernheim and Bernheim (1938).

In the presence of choline and arsenocholine the enzyme is completely inhibited under aerobic conditions by 0.001 M cyanide (Mann and Quastel, 1937). Under anaerobic conditions these substrates in the presence of liver extract reduce ferricyanide and this system is not inhibited by cyanide (Mann, Woodward and Quastel, 1938). It was shown by the latter workers that the dehydrogenase in the presence of choline or arsenocholine reduced cytochrome c; on shaking the reaction mixture with air the bands of reduced cytochrome c disappeared, but they reappeared on removal of the air. On the basis of these results it was concluded that the choline dehydrogenase system consists of choline, dehydrogenase, cytochrome c, cytochrome oxidase and oxygen. This system accounts for the cyanide-sensitivity of the aerobic oxidation of choline, and the cyanide-insensitivity of its anaerobic oxidation in the presence of ferricyanide.

Mann, Woodward and Quastel (1938) showed that arsenocholine has a lower affinity for the enzyme than has choline. The half-maximum rate of oxygen consumption is obtained with 1.2×10^{-3} M choline and with 8×10^{-3} M arsenocholine.

The dehydrogenase is inhibited by ammonium and trimethylammonium ions, and by betaine, indicating that the nitrogen group and not the primary alcohol group of the substrate is of primary importance for attachment to the enzyme (Mann, Woodward and Quastel, 1938). Haugaard (1946) showed that choline dehydrogenase is inactivated by an increase in oxygen pressure which is believed to be the result of the oxidation of essential -SH groups. It is also inhibited by stearic acid (Bernheim, 1940).

General Summary of Information on the Anaerobic Cytochrome-Linked Dehydrogenases

The dehydrogenases of this group are widely distributed in nature. They are, with the exception of the yeast enzyme, associated with insoluble cellular material. It is for this reason that they have not been obtained in highly purified form. The lactic enzyme of yeast is the only one of the group that is readily soluble and it has been purified. The natural acceptor is cytochrome c, and the cytochrome oxidase of the system is the point of action when inhibition is induced with cyanide. There is evidence indicating that succinic and lactic dehydrogenases require an unknown factor to link them with cytochrome c.

The cytochrome-linked dehydrogenases react with methylene blue under anaerobic and aerobic conditions. The reduction of the dye under anaerobic conditions and the consumption of oxygen under aerobic conditions are used to measure the activity of these enzymes. Likewise oxygen uptake in the presence of cytochrome c is used for this purpose.

The dehydrogenases of this group are often inhibited competitively by compounds structurally related to their substrates. Succinic and choline dehydrogenases are inactivated by reagents which react with -SH groups. Because of this the activities of these two dehydrogenases are believed to depend on -SH groups. The Michaelis constant, or substrate concentration required for half maximum activity, is 0.001 M for both succinic and α-glycerophosphate dehydrogenases, and 0.0012 M for choline dehydrogenase.

ANAEROBIC DEHYDROGENASES (Pyridine-nucleotide-linked)

MALIC DEHYDROGENASE

The discovery of malic dehydrogenase was made independently by Thunberg (1911) and by Battelli and Stern (1910b, 1911). These workers observed that malic acid is oxidized in the presence of animal tissues. That a coenzyme is required for the action was proved by Andersson (1933) for washed dried yeast, and by Holmberg (1934) for extracts of washed muscle. They showed that methylene blue is reduced in the presence of the extracts and malate, providing cozymase, now known as DPN, was added. This discovery made many of the earlier contributions of relatively little importance.

Malic dehydrogenase is found in brain, heart, liver, muscle, kidney and other animal tissues; in yeast and certain bacteria such as E. coli, and in higher plants (Elliott, 1941, Sumner and Somers, 1947). Green (1936b) found high concentrations in the tissues of the rat, rabbit and pigeon.

The fraction which precipitated from an extract of pig heart muscle at pH 4.6 was used by Green (1936b) for an extensive study of malic dehydrogenase.

Straub (1942b) isolated malic dehydrogenase by extracting acetone-dried pig heart muscle with 0.1 M phosphate buffer of pH 7.3. The extract was treated with $CaCl_2$, centrifuged, neutralized with trisodium phosphate and again centrifuged. The enzyme was precipitated with ammonium sulfate and ethanol, reprecipitated with ammonium sulfate, then with acetone and finally dissolved in water. The fact that the purified enzyme is water soluble served to clarify the issue as to the solubility of this dehydrogenase. The enzyme was not crystallized, but since it is an analog of lactic dehydrogenase, Straub showed the malic preparation to be of high purity by comparing its activity with the activity of crystalline lactic dehydrogenase.

The activity of the purified enzyme gradually declined on dialysis against distilled water and after two days 80 per cent of the activity was lost. It was not possible to reverse the inactivation by treatment with cysteine, cystine, reduced or oxidized glutathione or boiled extracts of muscle.

Determination of Activity

Straub (1942b) determined the activity of the purified enzyme by manometrically measuring oxygen uptake. The system consists of 0.3 mg. of pure diaphorase, 0.5 mg. DPN, 0.05 M L-malic acid, 0.1 M KCN and 1 mg. of methylene blue in the presence of a small amount of malic dehydrogenase. Maximum activity was obtained with 3 to 4 micrograms of the pure enzyme. Since cyanide was used to bind the oxalacetate, the oxygen consumption was proportional to the concentration of the enzyme.

The methylene blue method was used by Gale and Stephenson (1939) to measure the activity of a cell-free preparation obtained from E. coli (B. coli).

Potter (1946) reported a method for the determination of the total amount of malic dehydrogenase in tissue homogenates by measuring the rate of oxygen uptake in Warburg manometers. The system consists of 0.8 ml. of 0.1 M phosphate buffer of pH 7.4, 0.3 ml. of

0.5 M glutamate, 0.3 ml. of 0.5 M malate, 0.3 ml. of 4×10^{-4} M cytochrome c, 0.3 ml. of 0.1 M nicotinamide and 0.3 ml. of 1 per cent solution of DPN, plus the requisite amount of 2.5 per cent whole tissue homogenate and sufficient water to make a final volume of 3 ml.

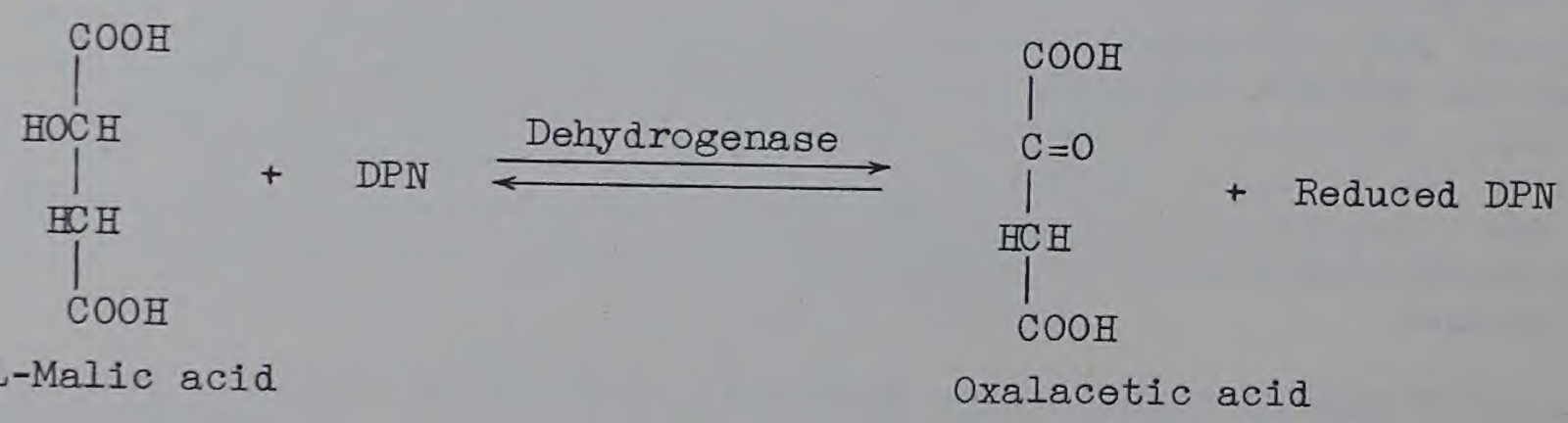
Banga and Szent-Gyorgyi (1937) showed that the inhibition of malic dehydrogenase by the oxalacetate formed in the reaction is prevented by addition of glutamate to the reaction mixture. Later work showed that the oxalacetate is converted to aspartate by transamination. Nicotinamide inhibits the breakdown of DPN and this tends to maintain the maximum rate of action (Mann and Quastel, 1941). These factors and the other necessary reagents were employed by Potter (1946) in amounts sufficient to make the dehydrogenase the limiting factor in the system. Under these conditions, when a homogenate of whole tissue is used, the potential dehydrogenase activity of the tissue is determined. As a tissue changes in function with time, an accurate measure of any corresponding changes in malic dehydrogenase activity can be obtained.

Potter (1946) studied the enzyme in mouse liver and brain; in rat liver, hepatoma, brain and kidney; in human kidney and in pigeon breast muscle. Rat hepatoma contained less enzyme than any of the other tissues. McShan, Erway and Meyer (1948) reported the changes in the activity of the dehydrogenase in the lutein and other ovarian tissues of the rat during pregnancy and lactation. The activity of the lutein tissue is highest during pregnancy when the corpora are in the functional state.

Specificity and Mechanism of Action .

Malic dehydrogenase is specific for natural L-malic acid (Green, 1936b). It is inactive toward D-malate and it does not oxidize maleic and dihydroxymaleic acids. Results reported by Mehler et al. (1947) indicate that malic dehydrogenase and also the lactic enzyme are not exclusively specific for DPN; they show slight activity with TPN. Their degree of aspecificity for the two coenzymes with various tissues is shown in Table II.

The complete malic system according to present evidence consists of malate, dehydrogenase, DPN, DPN-cytochrome c reductase, cytochrome c, cytochrome oxidase and oxygen (Potter, 1946). The overall reaction is represented as follows:



L-Malic acid — Oxalacetic acid

The activated hydrogen from the malate reduces DPN and the oxidation is completed through the cytochrome system. Mann and Quastel (1941) obtained the maximum rate of oxygen uptake with 0.03 M and half-maximum with 0.01 M malate. In the presence of cyanide one molecule of malic acid is oxidized per atom of oxygen consumed and the reaction results in the formation of oxalacetate (Green, 1936b). The dinitrophenylhydrazone of oxalacetate was prepared from the flask contents when semicarbazide was used as fixative. On addition of aniline citrate to the reaction mixture, carbon dioxide equivalent to the theoretical amount of oxalacetate formed was liberated.

The preparation of Green (1936b) catalyzed the oxidation of malate in the presence of DPN, methylene blue and cyanide. Substances such as adrenaline or adrenochrome, lactoflavin and pyocyanine can replace methylene blue in the system. Yellow enzyme gave little oxygen uptake, probably due to its slow oxidation. It required 0.25 M cyanide to prevent inhibition by the oxalacetate formed, and cyanide is more desirable in this respect than is hydrazine and semicarbazide. As indicated above, Banga and Szent-Gyorgyi (1937) showed that glutamate also prevents the inhibition of malic dehydrogenase by oxalacetate.

Dixon and Zerfas (1940) studied the conditions required for a wide range of acceptors to function with malic dehydrogenase. These acceptors fell into three groups: (I) those which require both DPN and flavoprotein, (2) those which require only DPN and (3) those which do not use either DPN or flavoprotein. Included in the latter group are substances not found naturally in tissues, such as alloxan, iodine, hydrogen peroxide and dibromophenolindophenol. This indicates that DPN is not necessary in the malic dehydrogenase system for all acceptors, but is one of several which reacts directly with the substrate.

The equilibrium of the malate system lies far to the left and as a result oxalacetate has a tendency to be reduced to malate by reduced DPN (Euler, Adler and Gunther, 1937b). This was shown for animal tissues and for yeast and it indicates that the system is reversible. The E_0' for the malate-oxalacetate system was shown by Laki (1937) to be -0.169 at pH 7.0 and 37° C. and -0.102 by Lehmann and Jorgensen (1939). Optimum activity is expressed over the range of pH 8.0 to 9.0.

Relationship of Malic to Lactic Dehydrogenase

Malic and lactic dehydrogenases have a number of properties in common and their distribution in tissues is very similar. Das (1937a) believed they were identical because under the proper conditions the rates of methylene blue reduction by malate and lactate were always about the same. Other workers have shown, however, that two distinct enzymes are involved. Green (1936b) found that the ratio of their activities is variable, depending on the source from which the enzyme preparations are obtained. A lactic dehydrogenase preparation obtained from E. coli was found to be inactive in the presence of malate. The purified malic dehydrogenase of Straub (1942b) does not catalyze the oxidation of lactic acid. Finally, malic acid is not oxidized by the crystalline lactic dehydrogenase of Straub (1940).

According to Green (1936b) oxalacetate is the most effective of any known inhibitor of malate oxidation. Calculations based on the limiting oxygen uptake of the system in the absence of cyanide as fixative indicate that 0.001 M will inhibit completely. Pyruvate and acetoacetate are effective inhibitors. Malonate, tartronate, oxalate and iodoacetate inhibit in concentrations above 0.03 M. Malic dehydrogenase is markedly inhibited by muscle adenylic and adenylpyrophosphoric acids. The latter compounds presumably compete with DPN for the dehydrogenase. Pyrophosphoric and arsenious acids, however, increase the oxygen uptake and the mechanism is not understood. This effect of these two compounds also holds for lactic and β-hydroxybutyric dehydrogenases. Inactivation is caused by increased oxygen pressure which is presumably due to the oxidation of essential -SH groups (Haugaard, 1946).

β-HYDROXYBUTYRIC DEHYDROGENASE

Wakeman and Dakin (1909) showed that oxygen is consumed when acetoacetate is formed from β-hydroxybutyrate in the presence of aqueous extracts of dog liver. Among other early workers Thunberg (1920) found that frog muscle reduces methylene blue in the presence of β-hydroxybutyric acid. The reduction of methylene blue by washed heart muscle in the presence of this acid was shown by Banga, Laki and Szent-Gyorgyi (1933) to be activated by the same coenzyme preparation as was required for the oxidation of lactate.

According to Green (1940) β-hydroxybutyric dehydrogenase is limited to a few tissues such as heart, liver and kidney, with heart the richest source. Quastel and Wooldridge (1925) showed that it is present in some bacteria.

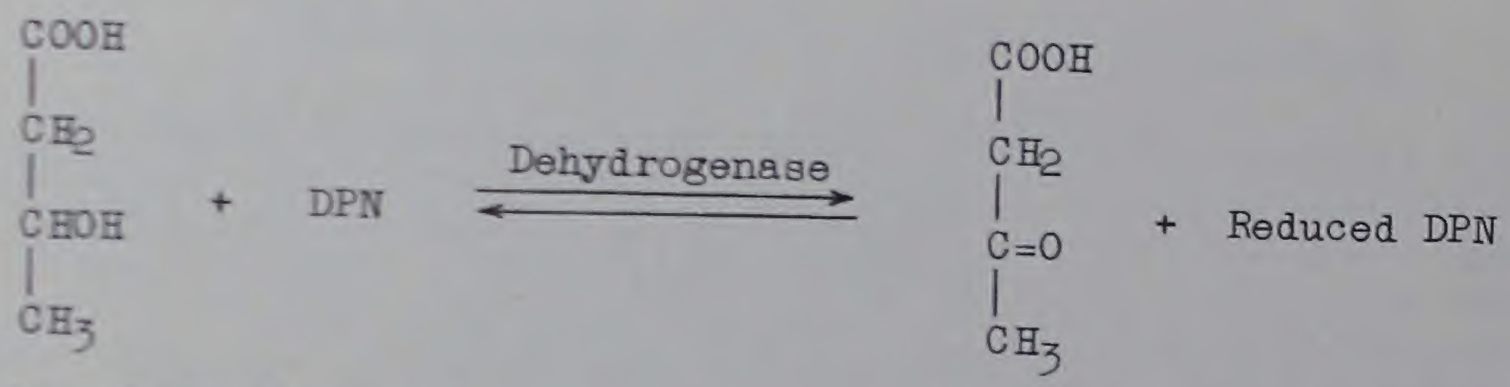
Because of the limited distribution of this dehydrogenase it is believed to account for only a small part of the total oxidations. It probably functions in the metabolism of food materials, since oxidation of higher fatty acids results in the formation of β-hydroxybutyric acid. Quastel and Wheatley (1935) and Krebs and Johnson (1937) made extensive studies of β-hydroxybutyrate and acetoacetate metabolism in animal tissues. It was suggested by the latter workers that the β-hydroxybutyrate-acetoacetate system can play a part in cellular hydrogen transport.

Green, Dewan and Leloir (1937) used the pig heart muscle preparation of Green and his coworkers for a comprehensive study of β-hydroxybutyric dehydrogenase. This preparation contained diaphorase and malic and lactic dehydrogenases.

Specificity and Mechanism of Action

The enzyme catalyzes the dehydrogenation of L-β-hydroxybutyrate. Compounds such as β-hydroxypropionate, α- and γ-hydroxybutyrate, crotonate, butyrate and acetate are not oxidized significantly. Lang (1942) observed dehydrogenation with DL-β-hydroxybutyric, -valeric, -caproic, -enanthic, - caprylic and -pelargonic acids. The dehydrogenase is specific for DPN and is inactive with TPN.

The catalytic dehydrogenation of L-β-hydroxybutyric to acetoacetic acid is represented by the following equation:



L-β-Hydroxybutyric acid — Acetoacetic acid

The hydrogen is accepted by DPN and is transferred to oxygen by the cytochrome system. It has been shown that one atom of oxygen is taken up for each molecule of substrate oxidized. For each atom of oxygen consumed one molecule of carbon dioxide was liberated when the hydrazone of acetoacetate was decomposed with alkali to acetone and hydrazine. The dinitrophenylhydrazone of acetone has been isolated and identified. One-half the theoretical oxygen uptake occurred with the DL-compound (Green, Dewan and Leloir, 1937).

Low concentrations of cyanide inhibit the β-hydroxybutyric dehydrogenase system, but oxygen consumption is restored by adding methylene blue, flavin or adrenochrome. These results are explained by the fact that the complete system consists of β-hydroxybutyrate, dehyd genase, DPN, the cytochrome system and oxygen. The cyanide inhibits the cytochrome oxidase of the system whereas the carriers permit the system to function without this enzyme.

The maximum initial rate of oxygen uptake is obtained with 0.07 M substrate and half-maximum with 0.005 M without fixative. Only with low concentrations of DPN is the rate proportional to the amount of this factor present. Little increase in the rate of oxygen uptake occurs when a fixative such as hydrazine is added, but a constant rate is maintained for a longer time. The pH of optimum activity is 7.3.

Properties and Inhibition

Green, Dewan and Leloir (1937) found the E_0' to be -0.282 for the β-hydroxybutyrate-acetoacetate system. Hoff-Jorgensen (1938) obtained a value of -0.293 at pH 7.0 and 38° C. This is more negative than was found for the lactate-pyruvate and malate-oxalacetate systems.

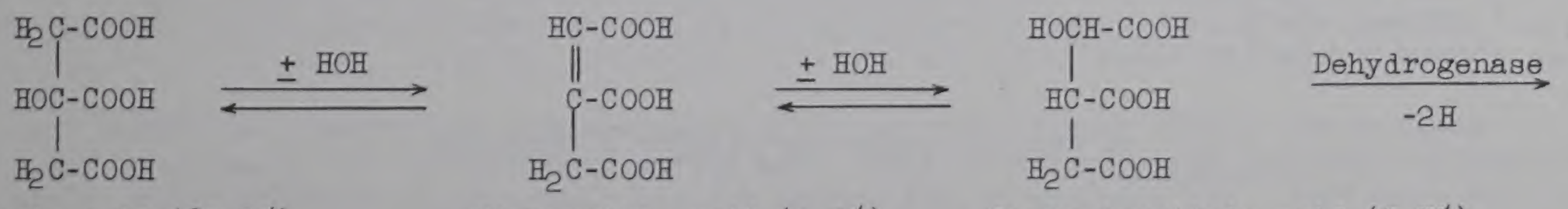
The most striking difference between these systems is the strong inhibition of the lactic and malic enzymes by their end-products of oxidation, pyruvate and oxalacetate, respectively, as compared to the negligible inhibition of β-hydroxybutyric dehydrogenase by acetoacetate. Green et al. (1937) suggested that this difference may be explained on the basis of the presence of more reduced DPN in the latter system than is ordinarily present in the lactic and malic systems. This is dependent on the proper differences in the oxidation-reduction potentials of these systems including that of DPN-reduced DPN.

β-Hydroxybutyric dehydrogenase is inhibited to a significant degree by acetoacetate, malonate, oxalacetate, pyruvate and arsenite. It is completely inhibited by saturation with n-octyl alcohol and markedly by 0.001 M adenylic acid. It is not inhibited by urethane. As with lactic and malic dehydrogenases, pyrophosphate causes an increase in oxygen uptake. Its effect on this enzyme is actually one of stabilization rather than acceleration in that the rate is maintained for a longer time (Green, Dewan and Leloir, 1937).

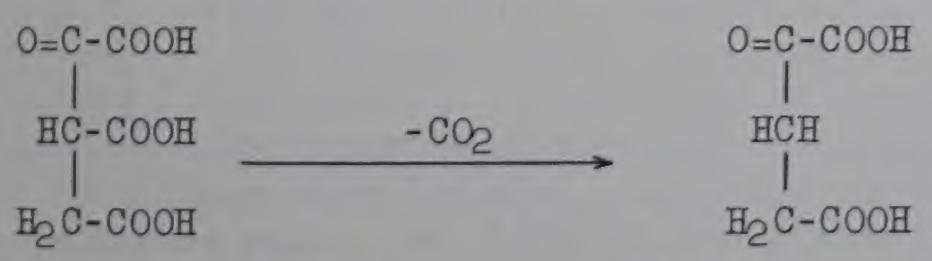
ISOCITRIC DEHYDROGENASE (Citric Dehydrogenase)

Battelli and Stern (1910b, 1911) first showed that citric acid is oxidized aerobically by various animal tissues. Thunberg (1920) was the first to observe that methylene blue is reduced by citrate in the presence of washed frog muscle and this was later shown to be true for most animal tissues. Thunberg (1929) prepared an alkaline phosphate extract of cucumber seed which in the presence of citrate reduced methylene blue. Andersson (1933) and Wagner-Jauregg and Rauen (1935a) used this extract to study the mechanism of action of the enzyme. Bernheim (1928b) obtained a water soluble preparation by precipitating the enzyme from an aqueous extract of acetone-dried liver with ammonium sulfate. This preparation in the presence of citrate reduced methylene blue. It seemed to be specific for citrate since reduction was not obtained with lactate, malate, succinate and various other compounds.

The catalytic oxidation of citrate by tissues was clarified by the important work of Martius (1937, 1938) in which he showed that α-ketoglutarate is formed from citrate in the presence of liver mince. His results indicate that the oxidation of citrate is represented by the following equations:



Citric acid (89.2%) — Cisaconitic acid (3.1%) — Dextroisocitric acid (7.7%)



Oxalsuccinic acid (α-Keto-β-carboxy-glutaric acid) — α-Ketoglutaric acid

The formation of isocitric from citric acid through the intermediate, cisaconitate, is nonoxidative and the equilibrium is catalyzed by an enzyme known as aconitase. It has not been shown definitely, however, that only one enzyme is involved in this equilibrium. Aconitase is similar to fumarase in stability and sensitivity to various reagents but they are not identical. Martius (1938) and Johnson (1939) studied the properties of aconitase and reported that at pH 7.0 the equilibrium values for citrate, cisaconitate and isocitrate are 80, 4 and 16 per cent respectively. According to later work by Martius and Leonhardt (1943), the percentages of the three acids present at equilibrium are 89.2, 3.1 and 7.7.

The isocitrate formed by the equilibrium established by aconitase is catalytically dehydrogenated to oxalsuccinate, which makes it evident that the enzyme acts on isocitric and not on citric acid. It is known, therefore, as isocitric dehydrogenase and aconitase must be present for it to act in the presence of citrate.

Distribution, Determination and Mechanism of Action

According to Adler, Euler, Gunther and Plass (1939) isocitric dehydrogenase has been found in all animal tissues studied; the highest concentrations are present in heart, liver, kidney and adrenal gland. Euler, Adler, Gunther and Elliot (1939) found it in higher plants and yeast. Bacteria and molds metabolize citrate and this probably takes place through the oxidation of isocitrate.

The activity of isocitric dehydrogenase can be measured by the Thunberg method, using methylene blue, or by the determination of oxygen consumption in Warburg manometers. Adler et al. (1939) determined the activity by using enzyme equivalent to 4 mg. of liver powder, 20 micrograms of TPN, 0.8 micrograms of lactoflavin, 0.5 ml. of 1 per cent methylene blue, 0.25 ml. of pH 7.66 veronal-acetate buffer and 5 mg. of isocitric acid in a total volume of 2.25 ml. Phosphate buffer cannot be used since phosphate ion inhibits the enzyme. Oxygen uptake was measured as an index of the activity.

Ochoa (1948) determined the activity of isocitric dehydrogenase by measuring, spectrophotometrically, the rate of reduction of TPN in the presence of the enzyme, Mn ions and an excess of isocitric acid. The enzyme was extracted from washed, acetone-dried pig heart with phosphate solution. It was recovered from this extract by precipitation with ammonium sulfate and partially purified by reprecipitation and by adsorption of inactive material with alumina gel. The activity of the dehydrogenase was increased four times over that of the initial extract.

Adler, Euler, Gunther and Plass (1939) made an extensive study of isocitric dehydrogenase. They prepared it free of aconitase from acetone-dried pig heart muscle. The enzyme was precipitated from the aqueous extract at 0° C. with 75 per cent acetone. This precipitation was repeated and the material was dried with acetone and ether. The enzyme was stable in the dry form.

Adler et al. (1939) showed that isocitric dehydrogenase specifically activates the naturally occurring dextroisocitric acid. The system functions in the presence of TPN, but not with DPN. The affinity of the enzyme for the substrate is extremely high, since the rate of oxidation was optimum in the presence of 1.25×10^{-5} M substrate. The Michaelis constant is therefore less than 1.25×10^{-5} M which is one of the lowest known values for oxidizing enzymes. The pH of optimum activity is 7.0 to 7.5 and below pH 6.5 the rate falls off rapidly.

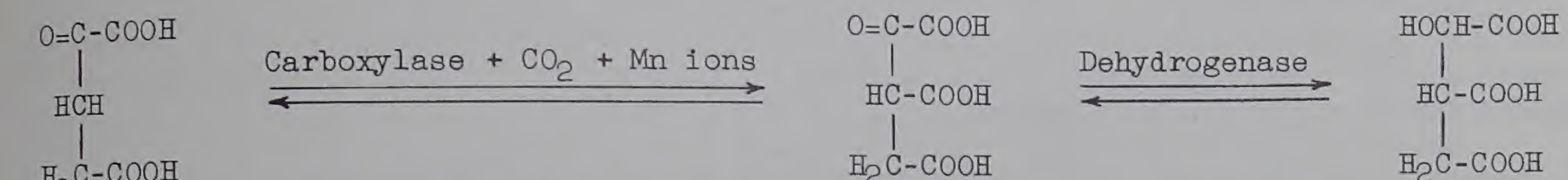
Ochoa (1945) showed that cytochrome reductase links the isocitric dehydrogenase system with cytochrome c, since the reductase catalyzes the reoxidation of reduced TPN by cytochrome c. Adler et al. (1939) had previously demonstrated that the system requires flavinenzyme. For each mole of isocitrate oxidized, one-half mole of oxygen was consumed and one mole of carbon dioxide was formed. The reaction product was isolated and shown to be α-ketoglutarate.

With low dilutions of the dehydrogenase the activity was not proportional to enzyme concentration. It appeared that this might be due to the presence of an activator in the preparation in minimum concentration. This was shown to be the case in that Mn ions restored the activity completely, whereas partial restoration was obtained with Mg ions. The optimum concentration of Mn ions is 5×10^{-4} M, and for Mg ions it is 2.5×10^{-3} M.

Reversibility of the Reaction

The overall reaction appeared to be irreversible, but it was not possible to decide whether the primary dehydrogenation of isocitrate is irreversible since the decomposition of oxalsuccinate is extremely rapid. Results obtained by Ochoa and Weisz-Tabori (1945) served to clarify this issue. In a study of carbon dioxide fixation they found an enzyme in pig heart muscle, oxalsuccinic carboxylase, which catalyzes the equilibrium between oxalsuccinate and α-ketoglutarate. This equilibrium lies far in the direction of decarboxylation. Reversibility of the reaction was demonstrated in the presence of isocitric

dehydrogenase and reduced TPN. The oxalsuccinate formed by carboxylation is reduced to dextroisocitrate by reduced TPN. The carboxylase requires the presence of Mn ions. Ochoa (1948) made a further study of the mechanism of this reaction and found the equilibrium constant for the overall reaction to be 1.3×10^{-4}. This also indicates that the equilibrium lies far toward decarboxylation or the formation of α-ketoglutarate. The reversibility of the decarboxylation was confirmed by Grisolia and Vennesland (1947). The reactions are represented as follows:



α-Ketoglutaric acid — Oxalsuccinic acid — Dextroisocitric acid

The results given above show that the catalytic dehydrogenation of isocitrate by isocitric dehydrogenase is clearly reversible. If aconitase is present, citrate is also formed due to the equilibrium established. The isocitrate-ketoglutarate system is, according to the citric acid cycle of Krebs, part of a catalytic oxidative system concerned with the breakdown of carbohydrate. The α-ketoglutarate can react in this cycle to form succinate. The above results also show that this system may play an important part in the biological utilization of carbon dioxide.

In concentrations of 0.01 and 0.001 M iodoacetate inhibited 97 and 75 per cent, respectively. This inhibition is effected by action with the isocitric dehydrogenase and probably by interaction with -SH groups. The strong inhibition by pyrophosphate results from binding Mn and Mg ions. Cyanide, malonate, fluoride and oxalate do not inhibit (Green, 1940).

GLUCOSE-6-PHOSPHATE DEHYDROGENASE

(Hexose-6-phosphate Dehydrogenase, Zwischenferment, Robison Ester Dehydrogenase)

Barron and Harrop (1928, 1929) showed that in the presence of methylene blue or methemoglobin, glucose is oxidized by mammalian red blood cells. Warburg and Christian (1931a,b) found that a clear solution prepared from laked or cytolyzed erythrocytes would not catalyze the oxidation of glucose, but that in the presence of methylene blue or methemoglobin it would oxidize the Robison ester, glucose-6-phosphate. The ability of the cells to phosphorylate glucose was apparently lost by cytolysis. By adsorption on aluminum hydroxide the active agent was separated from the solution and found to consist of two factors, a ferment and a coferment. These two factors, in the presence of hexosemonophosphate, caused oxygen uptake which was increased by the addition of methylene blue. In the light of later work the two preparations must have contained yellow enzyme, diaphorase or similar mediators to account for the reaction with methylene blue or oxygen.

The work of Warburg and Christian (1931b) on the mechanism of hexosemonophosphate oxidation is important because it resulted in the discovery of TPN and yellow enzyme, which in turn led to the development of our present knowledge of coenzyme systems. Their coferment I was later shown to be TPN, and their coferment II obtained from blood cells was later reported to be a mixture of coferment I and glutathione. Their zwischenferment, which catalyzed the oxidation of hexosemonophosphate, is now known as glucose-6-phosphate dehydrogenase.

Glucose-6-phosphate dehydrogenase is found in yeast and blood cells; its distribution in other animal tissues is not known. Wagner-Jauregg, Moller and Rauen (1935) prepared it from frog muscle.

Preparation and Purification

Warburg and Christian (1932) prepared the dehydrogenase from an extract of dried bottom yeast. The enzyme was separated by diluting the extract with water saturated with carbon dioxide.

Red blood cells of the rat can also serve as a ready source of this enzyme. The corpuscles are washed with 0.9 per cent NaCl, cytolyzed with water and kept at 0° C. A crystalline precipitate of hemoglobin forms and carries down the enzyme which is recovered by centrifuging, followed by elution with water. According to Theorell (1934) the isoelectric point of the enzyme prepared from rat blood is pH 5.85.

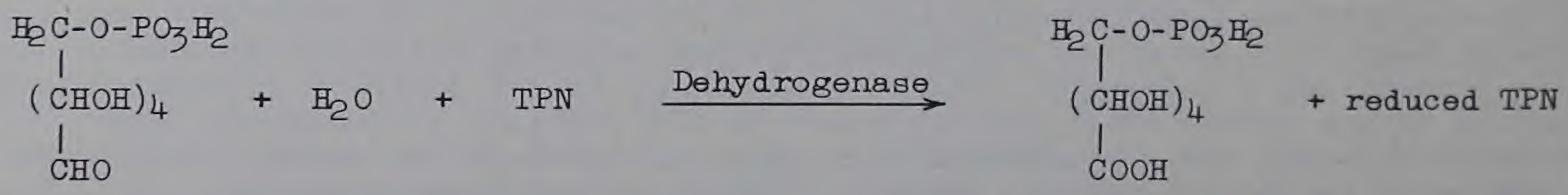
Negelein and Gerischer (1936) obtained highly purified dehydrogenase from an extract of dried bottom yeast. In their method acid is added to pH 4.6, the inactive precipitate that forms is separated, and the solution is treated at pH 9.0 and 30° C. to inactivate certain destructive enzymes. The solution is cooled, adjusted to pH 4.8 and the enzyme is precipitated with ammonium sulfate. The precipitate is dissolved in water, made 30 per cent saturated with ammonium sulfate, and heated to 40° C. to give an inactive precipitate which is discarded. The solution is dialyzed, the enzyme is precipitated with ethanol, dissolved, and precipitated twice at pH 4.8, its isoelectric point. The enzyme becomes insoluble at the isoelectric point only after most of the impurities are removed.

The final product was dried from the frozen state. It was almost colorless, was soluble in water except at the isoelectric point and the yield was 8.5 per cent of the dehydrogenase content of the original extract. The enzyme is unstable in solution at 0° C. as inactivation occurred in 24 hours, but it was stable in 30 per cent saturated ammonium sulfate solution. The nitrogen content was 13.3 per cent.

Negelein and Gerischer (1936) determined the activity of their purified glucose-6-phosphate dehydrogenase by measuring the oxygen uptake manometrically. The system consists of 14 mg. hexosephosphate, 0.02 mg. TPN, 0.5 mg. KCN, sufficient dehydrogenase to cause an oxygen uptake of 2 to 4 c.mm. per minute, and 10^{-8} moles yellow enzyme dissolved in 3.45 ml. of 0.01 M phosphate buffer. An oxygen uptake of 350 c.mm. of oxygen per minute was obtained with 1 mg. of the purified enzyme.

Specificity and Mechanism of Action

The dehydrogenase is specific for glucose-6-phosphate and does not attack fructose phosphate and hexosediphosphate. It is highly specific for TPN and is inactive with DPN. The reaction is represented as follows:



Glucose-6-phosphate (hydrate) — 6-Phosphogluconic acid

Warburg and Christian (1932) showed that glucose-6-phosphate dehydrogenase prepared from yeast catalyzes the oxidation of glucose-6-phosphate in the presence of purified coferment, TPN, of blood cells, and yellow enzyme obtained from bottom yeast. The TPN receives the hydrogen and passes it to the yellow enzyme which is reoxidized by oxygen or methylene blue. The rate of reaction decreases as reduced TPN is formed. This is due to the fact that the reduced TPN competes with oxidized TPN for the enzyme as they seem to have equal affinity for the dehydrogenase. The TPN is highly active in the presence of excess dehydrogenase and yellow enzyme.

It was shown by Warburg, Christian and Griese (1935) that one mole of glucose-6-phosphate is oxidized for each half mole of oxygen consumed when the reaction proceeds to

completion in the presence of purified enzyme. From this it was concluded that phosphohexonic acid is formed, and 6-phosphogluconic acid was isolated from the reaction mixture of a large-scale experiment. Reversibility of the system has not been demonstrated. The 6-phosphogluconic acid is catalytically oxidized in the presence of TPN to the corresponding keto acid by phosphohexonic acid dehydrogenase.

Phosphate inhibits glucose-6-phosphate dehydrogenase. This is apparently due to competition with the coenzyme for the enzyme. According to Dickens and McIlwain (1938) iodide in low concentrations inhibits this system, and Euler and Adler (1935) found that the dehydrogenase is strongly inhibited by heavy metals such as Cu ions. Hass (1944) showed that glucose-6-phosphate dehydrogenase is inhibited competitively by low concentrations of atabrine. Altman (1946) found that it is effectively inhibited by sulfonamides.

α-GLYCEROPHOSPHATE DEHYDROGENASE (DPN)

The α-glycerophosphate dehydrogenase discussed in this section acts in the presence of DPN. It is different from the dehydrogenase of Green which catalyzes the direct reaction of the same substrate with cytochrome c. The DPN-linked enzyme is also different from triosephosphate dehydrogenase. This is shown by the fact that it is insensitive to iodoacetate, as compared to the sensitivity of the triosephosphate enzyme to this reagent.

The DPN-linked dehydrogenase is found in muscle, kidney, liver and brain of the rat, and in rabbit muscle (Euler, Adler and Gunther, 1937a). It is also present in heart muscle.

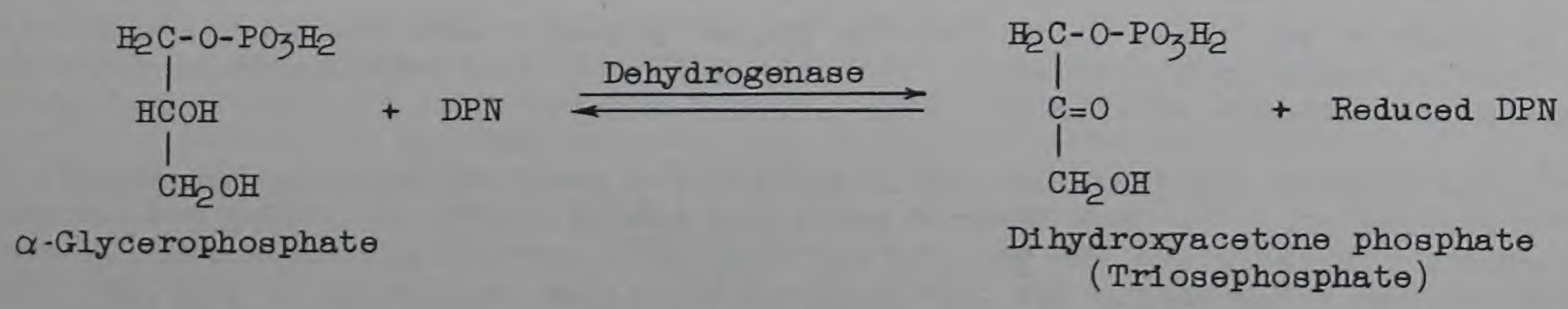
A soluble extract of this dehydrogenase was obtained from cucumber seed (Wagner-Jauregg and Rauen, 1935b). A soluble preparation has also been obtained by grinding and centrifuging plasmolyzed yeast (Ogston and Green, 1935, 1936). This yeast enzyme oxidized α-glycerophosphate and apparently it was not linked with the cytochrome system.

Euler, Adler, Gunther and Hellstrom (1937) showed that an extract of acetone dried muscle powder causes the dehydrogenation of glycerophosphate in the presence of DPN. The oxidation of α-glycerophosphate was also studied by Euler, Adler and Gunther (1937a). They used the pH 5.5 insoluble fraction from an alkaline phosphate extract of minced rat muscle. This preparation contained α-glycerophosphate and lactic and malic dehydrogenases. Results obtained on fractionating muscle extract showed that α-glycerophosphate dehydrogenase is distinct from the latter two enzymes (Adler, Euler and Hughes, 1938).

The activity of the enzyme was determined by the Thunberg method (Euler, Adler and Gunther, 1937a). The reaction mixture consists of 0.25 ml. of 0.5 M phosphate buffer of pH 7.6, 0.5 ml. of 0.02 per cent methylene blue, 0.25 ml. of 0.5 M potassium glycerophosphate, 0.25 ml. of enzyme, 0.5 mg. of DPN, and 0.25 ml. of yellow enzyme. Adler, Euler and Hughes (1938) determined the activity by following the reduction of DPN spectrometrically.

α-Glycerophosphate dehydrogenase is highly specific for natural α-glycerophosphate. It does not activate the unnatural isomer or β-glycerophosphate. It requires DPN and is not active in the presence of TPN.

The α-glycerophosphate system is represented as follows (Adler, Euler and Hughes, 1938):



α-Glycerophosphate

Dihydroxyacetone phosphate (Triosephosphate)

According to Adler, Euler and Hughes (1938) the product of the dehydrogenation is dihydroxyacetone phosphate. The evidence for this is also summarized in the review by Elliott (1941). The dihydroxyacetone phosphate forms an equilibrium with glyceraldehyde phosphate and when these two compounds (triosephosphate) are added, the reduced DPN is rapidly reoxidized. Euler et al. (1937a) suggested that the principal function of this dehydrogenase is the catalysis of the reduction of triosephosphate to glycerophosphate in the presence of reduced DPN.

The system reduces methylene blue and also yellow enzyme in the presence of DPN. When other systems are not present to oxidize the reduced DPN, an equilibrium is established as indicated in the equation. The position of the equilibrium and the rate of reduction of coenzyme are dependent on the pH. Equilibrium constants were calculated from data obtained by spectrometrically following the reduction of the coenzyme. The constants found at pH 6.9, 7.6 and 8.1 are 6.3×10^4, 0.33×10^4 and 0.17×10^4, respectively.

Euler, Adler and Gunther (1937a) showed that a preparation made from rabbit muscle caused more rapid reduction of methylene blue with glycerophosphate in the presence of DPN than in its absence. The activity in the absence of DPN is due to the presence of Green's cytochrome-linked dehydrogenase since the preparation was shown not to be contaminated with DPN.

Adler, Euler and Hughes (1938) demonstrated that muscle extract in the presence of glycerophosphate and pyruvate oxidizes reduced DPN. Since the extract contained lactic dehydrogenase, the pyruvate was reduced to lactate. Because this reaction takes place in the presence of lactic and glycerophosphate dehydrogenases, it is, according to Adler, Euler and Hughes (1938), equivalent to the oxidation of glycerophosphate by mutase reported by Green, Needham and Dewan (1937). Meyerhof and Kiessling (1933, 1935) observed this dismutation and concluded that since this reaction is slow, another system, namely the dismutation between triosephosphate and pyruvate, is the main lactate-yielding reaction.

Another reaction in which glycerophosphate dehydrogenase takes part is the dismutation of two molecules of triosephosphate to give glycerophosphate and phosphoglycerate. This reaction takes place in the absence of a hydrogen acceptor, such as pyruvate, and in the presence of DPN and triosephosphate and glycerophosphate dehydrogenases.

Pyruvate and oxalacetate inhibit the reduction of methylene blue by the DPN-linked system of the muscle extract prepared by Euler, Adler and Gunther (1937a). This is because the reduced DPN reduces these compounds instead of the methylene blue since lactic and malic dehydrogenases are present in the preparation. The enzyme is not inhibited by cyanide and iodoacetate.

ALCOHOL DEHYDROGENASE

(Aldehyde Reductase)

The early work on alcohol dehydrogenase is of interest because the first recognition of an oxidizing enzyme as a dehydrogenase was the observation of Wieland (1913) that methylene blue is reduced in place of oxygen during the oxidation of alcohol by acetic acid bacteria. It was also the first oxidizing enzyme to be crystallized. The results obtained with various tissues and organisms previous to the discovery of coenzymes and yellow enzyme have, to a large extent, been superseded by the results of later work.

Various kinds of bacteria, molds, yeasts, higher plants, seeds and animal tissues are capable of catalytically oxidizing alcohol (Elliott, 1941). The dehydrogenase of yeast, animal tissues and bacteria will be discussed in this order.

Crystalline alcohol dehydrogenase was prepared from yeast by Negelein and Wulff (1937). The maceration juice from baker's yeast was heated to 56° C., which precipitated inactive protein. The solution was made 35 per cent with acetone and the resulting precipitate was removed. The enzyme was precipitated by adding acetone to 50 per cent and dried *in vacuo*. The dry material was dissolved in water, and the more soluble protein impurities were removed by precipitating the enzyme with ammonium sulfate, first at 62 per

cent and then at 50 per cent saturation. It was next precipitated successively at pH 5.0 in the presence of alcohol concentrations of 64, 60 and 26 per cent. The enzyme, which precipitates slowly from 26 per cent alcohol, was dissolved in water and made 27 per cent saturated with ammonium sulfate. The dehydrogenase crystallized from this solution.

The enzyme was stable when kept in the dry form in the cold. It was highly active as shown by the fact that 0.001 mg. caused an uptake of 2.5 c. mm. of oxygen per minute which is equivalent to a Q_{O_2} of 150,000.

The activity of alcohol dehydrogenase was determined by Negelein and Wulff (1937) by measuring the oxygen uptake manometrically. The reaction mixture consists of 1 ml. of 0.15 M pyrophosphate buffer of pH 7.8, 0.3 ml. of 0.1 M semicarbazide solution, 0.3 ml. of 4×10^{-5} M yellow enzyme, 0.1 ml. of alcohol, 0.35×10^{-3} mg. of enzyme protein and 0.4 mg. of DPN. The semicarbazide was used to bind the aldehyde and prevent the reaction from coming to equilibrium. The Thunberg method can also be used to measure the activity.

Alcohol dehydrogenase is not highly specific, since normal propyl, isopropyl, secondary propyl, isobutyl, butyl, normal amyl and other alcohols are oxidized (Muller, 1934; Euler, Adler and Hellstrom, 1936). Hydroxy acids, sugars and polyhydroxy compounds are not activated. The enzyme is specific for DPN.

Dehydrogenase preparations of yeast in the presence of alcohol reduced methylene blue rapidly on addition of DPN and yellow enzyme. The activated hydrogen reduces the DPN, and yellow enzyme mediates the transfer of the hydrogen from the reduced DPN to methylene blue or oxygen (Euler and Adler, 1934, 1936). The oxidation results in the formation of acetaldehyde and the reaction is represented by the equation:

$$\begin{array}{l} CH_3 \\ | \\ CH_2OH \end{array} + \text{DPN} \rightleftharpoons \begin{array}{l} CH_3 \\ | \\ CHO \end{array} + \text{Reduced DPN}$$

Ethyl Alcohol — Acetaldehyde

Reversibility of this reaction was indicated by the visible reoxidation of leuco-yellow enzyme by acetaldehyde in the presence of the dehydrogenase and DPN (Euler and Adler, 1934, 1936). This was confirmed spectrophotometrically by Euler, Adler and Hellstrom (1936) and by Negelein and Wulff (1937) using pure enzyme. The reduction of aldehyde is the more rapid reaction and the dehydrogenase has much greater affinity for aldehyde than it has for alcohol. Maximum rate of DPN reduction was obtained with 0.3 M alcohol and maximum rate of reduced DPN oxidation with 2.5×10^{-3} M aldehyde.

Negelein and Wulff (1937) concluded, on the basis of kinetic studies, that the oxidation of alcohol to aldehyde involves the formation of a complex compound between the dehydrogenase and coenzyme. With low concentrations of enzyme the dissociation constant for the compound of dehydrogenase and DPN is 3×10^{-5}, whereas the constant for the dehydrogenase and reduced DPN is 9×10^{-5}. Thus the affinity of DPN for the enzyme is three times that of reduced DPN. As indicated above the dehydrogenase has much greater affinity for aldehyde than for alcohol.

Negelein and Wulff (1937) showed that on the basis of a molecular weight of 70,000, one molecule of the dehydrogenase in the presence of excess alcohol catalyzes the reduction of 17,000 molecules of DPN per minute at 20° C. In the presence of excess acetyldehyde and the enzyme 28,500 molecules of reduced DPN are oxidized per minute. This indicates that in the presence of low concentrations of alcohol, aldehyde is reduced many times faster than alcohol is oxidized. The equilibrium, therefore, lies far to the left. This makes it essential to fix the aldehyde with a reagent like semicarbazide in order to study the aerobic oxidation of alcohol. According to Euler, Adler and Hellstrom (1936) the equilibrium constant increases with decreasing pH because at pH 7.7, 7.0 and 6.4 it is 0.8×10^4, 1.9×10^4, and 7.8×10^4, respectively.

Euler, Adler and Hellstrom (1936) showed that in fermenting yeast acetaldehyde is reduced to alcohol by alcohol dehydrogenase in the presence of reduced DPN. The reduced DPN

presumably is formed by the dehydrogenation of triosephosphate to phosphoglycerate by triosephosphate dehydrogenase. The DPN forms the link between the two dehydrogenases and since, as was shown above, the equilibrium for the alcohol dehydrogenase system lies far to the left, alcohol is readily formed. In this coupled system the alcohol dehydrogenase is catalyzing the reduction of acetaldehyde and this is the reason it has sometimes been called acetaldehyde reductase.

The E'_o for the ethyl alcohol-acetaldehyde system was reported by Kalchar (1941) to be -0.163 V at 25° C. and pH 7.0. According to Euler and Hellstrom (1937) the pH of the isoelectric point of purified dehydrogenase of yeast is 5.2 as indicated by cataphoresis.

According to Dixon (1937) alcohol dehydrogenase is markedly inhibited by 0.001 M iodoacetate and also by 0.00033 M iodoacetate, providing the enzyme is incubated with the reagent for a short time. Cyanide does not inhibit. According to Euler and Adler (1935) the enzyme is strongly inhibited by Cu, Ag and Hg ions. This is also true for the crystalline enzyme. The activation of the system by reduced glutathione is probably due to removal of heavy metal ions.

Alcohol Dehydrogenase of Animal Tissues and Bacteria

The oxidation of alcohol by animal tissues was first studied by Battelli and Stern (1910c). They showed that liver is the richest source of the enzyme and kidney is second in activity. Other tissues are almost free of the enzyme. The alcohol dehydrogenase of animal tissues has been studied by Lutwak-Mann (1938). The enzyme was obtained from horse liver. After heating an extract of the liver to eliminate inactive protein, the enzyme was precipitated twice with acetone. Further purification was accomplished by fractionation with ammonium sulfate. It is stable in the dry form.

Bonnichsen and Wassen (1948) prepared alcohol dehydrogenase from horse liver by a modification of the method of Lutwak-Mann (1938). They obtained the enzyme in crystalline form after several fractionations and precipitations with ammonium sulfate and ethyl alcohol.

It was shown by Lutwak-Mann (1938) that DPN is required for the oxidation of alcohol in the presence of methylene blue. If a molecular weight of 70,000 is assumed, and 0.1 M alcohol and 0.15 mg. DPN per ml. are used, one molecule of the crystalline dehydrogenase converts 220 molecules of DPN per minute to reduced DPN (Bonnichsen and Wassen, 1948). Addition of cytochrome, cytochrome oxidase and diaphorase in the form of washed heart muscle resulted in rapid oxygen uptake (Lutwak-Mann, 1938). Ethyl alcohol was oxidized to acetaldehyde, and amyl and propyl alcohols were oxidized almost as fast as ethanol. α-Glycerophosphate and glycerol are not activated, and methyl alcohol has no effect on the oxidation of ethanol. Optimum activity with methylene blue was found to be at pH 7.6 and, according to Reichel and Kohle (1935), the optimum is pH 7.0 for the aerobic oxidation of propyl alcohol.

The dehydrogenase of animal tissues and bacteria is not inhibited by 0.01 M iodoacetate, whereas the enzyme of yeast as indicated above is inhibited by 0.001 M. This indicates that the enzyme of yeast is not identical with that of animal tissues and bacteria. Bernheim (1938) found that 10^{-4} M alloxan increases the rate of alcohol oxidation by liver suspensions, but this effect has not been explained.

DPN is required for the oxidation of alcohol by acetic acid bacteria (Lutwak-Mann, 1938). Results obtained with suspensions of Acetobacter suboxydans indicate that the coenzyme is present in the suspension and that it is removed by dialysis. The pH of optimum activity is 4.5. Methyl alcohol is not oxidized, but it inhibits the oxidation of ethanol.

GLUCOSE DEHYDROGENASE

Glucose dehydrogenase which catalyzes the oxidation of glucose to gluconic acid was discovered in mammalian liver by Harrison (1931). It is not identical with glucose oxidase found in Aspergillus niger by Muller (1928, 1931) and in Penicillium notatum by Birkinshaw

and Raistrick (1943) since the oxidase does not require coenzyme and does not react with methylene blue. The enzyme from Penicillium is also known as notatin.

According to Lynen and Franke (1941) glucose dehydrogenase occurs in the livers of various animals, such as the cow, horse, sheep and goat.

Harrison (1931) prepared the dehydrogenase by extracting acetone dried liver with water and recovered the enzyme by saturating the extract with ammonium sulfate. The precipitate was dissolved in water, dialyzed and filtered without loss of activity. Mann (1932) precipitated the enzyme by half-saturation with ammonium sulfate, but it was relatively inactive until an activator was added. The activator was obtained from a boiled aqueous extract of liver by precipitation with alcohol. This suggested that a coenzyme was required.

Harrison (1933) prepared glucose dehydrogenase that was relatively free of coenzyme. Acetone dried liver was extracted with water. The aqueous extract was dialyzed and an inactive precipitate was removed at pH 5.7. The dehydrogenase was precipitated by half-saturation with ammonium sulfate, reprecipitated, dried and stored *in vacuo*. The enzyme contained in this preparation, in the presence of glucose, reduced methylene blue very slowly until coenzyme was added.

The coenzyme was precipitated from the half-saturated filtrate by saturating it with ammonium sulfate. The precipitate was dissolved and heated in boiling water to coagulate the protein so as to separate it from the coenzyme which was recovered by evaporating the filtrate to dryness. After separation from the protein, the coenzyme was dialyzable and it was not precipitated with ammonium sulfate.

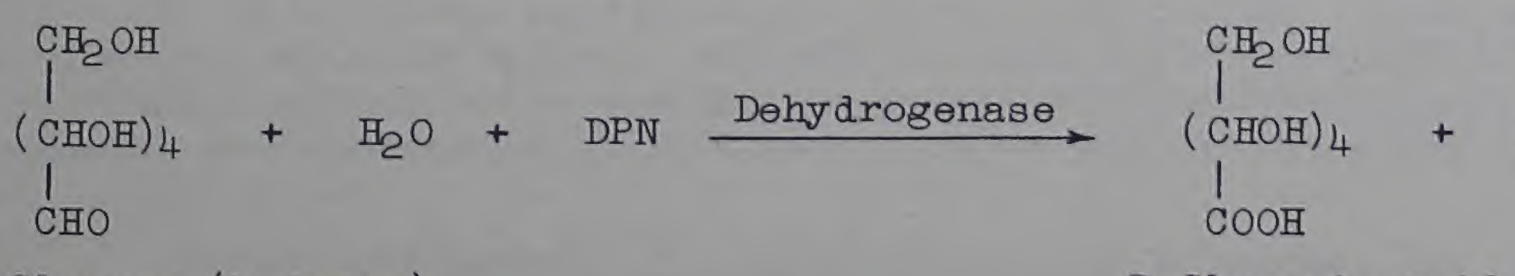
Andersson (1934) showed that cozymase can act as the coenzyme of glucose dehydrogenase and since cozymase was found to be present in the coenzyme preparation of Harrison, it was concluded that the enzyme requires cozymase or DPN. Purified dehydrogenase of liver is specific for DPN (Lynen and Franke, 1941). The reports of Das (1936) and Quibell (1938) that it reacts either with DPN or TPN are presumed to have been due to contamination of their preparations.

Specificity and Action

Glucose dehydrogenase is highly specific for D-glucose. It does not activate other sugars, such as fructose, galactose and arabinose.

The activity of glucose dehydrogenase can be determined by measuring the oxygen uptake manometrically (Schultze, Harrer and King, 1939). Glucose is the substrate and it reacts with methylene blue in the presence of the enzyme and DPN. The dye acts as mediator for oxygen uptake.

The oxidation of glucose to gluconic acid is represented as follows:



D-Glucose (hydrate) D-Gluconic acid

Harrison (1932) showed that approximately 0.5 mole of oxygen is consumed in the presence of methylene blue for each mole of glucose oxidized. The end-product was isolated from the reaction mixture as the calcium salt in 60 per cent yield and identified as D-gluconic acid. The maximum rate of reaction is obtained with 0.25 M and half-maximum with 0.07 M glucose. This indicates that the enzyme has low affinity for its substrate. The activity was constant from pH 6.0 to 8.0.

Glucose dehydrogenase oxidizes glucose through action with DPN, cytochrome c and cytochrome oxidase (Harrison, 1931). The results with cytochrome c were not confirmed by Ogston and Green (1935). However, Hawthorne and Harrison (1939) showed that cytochrome c takes part in the reaction, but it requires an additional factor to link it with DPN so the system will function. This factor is present in heart muscle and in cytochrome oxidase preparations made from heart muscle.

This enzyme is strongly inhibited by low concentrations of heavy metal ions such as 1.8×10^{-5} M Cu ions (Euler and Adler, 1935). It is not inhibited by cyanide, fluoride, iodoacetate and toluene.

Harrison (1933) reported that the activity of glucose dehydrogenase depends on an aldehyde group in the enzyme molecule. Evidence for this conclusion is: (1) The enzyme is irreversibly and completely inactivated on incubation with xanthine dehydrogenase which is known to activate aldehydes. A hydrogen acceptor such as oxygen or methylene blue must be present for this inactivation to take place, but DPN is not required. (2) Reversible inhibition due to competition with glucose for the enzyme is also caused by 0.01 M bisulfite at pH 6.0. Inhibition was not obtained at pH 7.6, since aldehyde-bisulfite compounds are decomposed in alkaline solution.

According to Yudkin (1933, 1934) there is a glucose dehydrogenase in E. coli which requires DPN. Quastel and Wooldridge (1925, 1927) and Quastel and Whetham (1925) showed that in the presence of glucose certain bacteria reduce methylene blue. The dehydrogenase of bacteria, however, differs in certain respects from the enzyme of liver. It is inhibited by toluene whereas the liver enzyme is not and it is active with low concentrations of glucose, such as 10^{-4} M, as compared to 0.25 M for the tissue enzyme.

FORMIC DEHYDROGENASE

Wishart (1923) reported that phosphate extract of rabbit liver will catalyze the oxidation of formate as shown by the methylene blue test. It is indicated in the review by Elliott (1941) that formic dehydrogenase is found in frog muscle and in the liver and muscle of the guinea pig. It occurs in yeast and molds. It is widely distributed in bacteria and in plant tissues in which it is highly active.

Higher Plants as Source of Dehydrogenase

Fodor and Frankenthal (1930) showed that formic dehydrogenase can be extracted with water from plant tissues such as peas and cereal grains. The enzyme was purified by precipitation with acetone and extraction of the precipitate with pH 6.0 phosphate solution, followed by treatment of the solution with kaolin. When formate was added to this preparation an increase in methylene blue reduction occurred. A dialyzed phosphate extract of peas prepared by Andersson (1934), in the presence of formate, reduced methylene blue only when coenzyme was added. The dehydrogenase was shown to be specific for DPN by Adler and Sreenivasaya (1937). Yellow enzyme caused increased oxygen uptake, but did not affect the reduction of methylene blue. Spectrophotometric observations showed that the enzyme in the presence of formate reduces 85 per cent of the DPN. Carbon dioxide in the presence of the enzyme and under normal pressure did not reoxidize the reduced DPN, indicating that the reaction is not easily reversible. The overall reaction is represented as follows:

$$\underset{\text{Formic acid}}{HCOOH} + \text{Acceptor} \longrightarrow \underset{\text{Carbon dioxide}}{CO_2} + \text{Reduced Acceptor}$$

Fodor, Frankenthal and Kuk (1930) did not obtain cyanide inhibition of methylene blue reduction with the plant enzyme. Later Adler and Sreenivasaya (1937) studied the formic dehydrogenase of the green pea and found that both methylene blue reduction and oxygen consumption are almost completely inhibited by 0.001 M cyanide. The reason for this difference in results is not clear. This is the only coenzyme-linked dehydrogenase reported to be inhibited by cyanide.

Bacteria as Source of Dehydrogenase

Suspensions of resting bacteria or preparations in which other dehydrogenases were inactivated by treatment with toluene have been used for the study of formic dehydrogenase. Quastel and coworkers (1925, 1927) showed that bacteria such as E. coli (Escherichia coli) reduce methylene blue in the presence of formate and Cook and Stephenson (1928) reported that they cause oxygen uptake with formate. Washed suspensions of the bacteria reduce the dye faster in the presence of formate than with the other compounds tested except sugars.

Stickland (1929) demonstrated that when E. coli are digested with trypsin for as long as two weeks, the dehydrogenase activity increases as much as five times in the first few days. The enzyme, which was associated with insoluble material, reduced methylene blue in the presence of formate, but did not take up oxygen, suggesting that the digestion destroyed cytochrome oxidase.

Gale (1939) prepared formic dehydrogenase from E. coli in the form of a fine sediment and studied the enzyme in some detail. The results of Stickland (1929) using tryptic digestion were confirmed. It was shown that the dehydrogenase reacts with oxygen through the cytochrome system. This reaction is inhibited by cyanide. According to Cook, Haldane and Mapson (1931) low concentrations of cyanide inhibit the aerobic oxidation of formate by E. coli, but not the reduction of methylene blue. Inhibition by other reagents is discussed in the review by Elliott (1941). The enzyme does not require DPN or TPN. In the light of these results it appears that the formic enzyme of E. coli should be classed with the cytochrome-linked dehydrogenases. It follows from this that the bacterial enzyme is distinct from the DPN-linked dehydrogenase of higher plants.

The enzyme in the digested sediment used by Gale (1939) is inactivated by oxygen in the presence of formate. Reactivation occurred on anaerobic incubation. Increasing the oxygen tension and the action of cyanide accelerated the inactivation, whereas lowering the oxygen tension and the presence of methylene blue inhibited the inactivation. It was suggested on the basis of these results that the dehydrogenase reacts with oxygen (1) directly, which leads to inactivation, and (2) indirectly through the carrier system, which diverts the oxygen and protects the enzyme.

Cook and Alcock (1931) found that E. coli in the presence of formate show optimum activity toward methylene blue from pH 7.0 to pH 9.5 when phosphate buffer is used. The optimum is at pH 9.5 with borate buffer. Oxygen uptake was greatest at pH 6.0. The concentration of formate for maximum methylene blue reduction is 10^{-4} M and for half-maximum it is 3×10^{-5} M.

GLUTAMIC DEHYDROGENASE is discussed in the chapter on NITROGEN METABOLIZING ENZYMES, and LACTIC and TRIOSEPHOSPHATE DEHYDROGENASES are presented in the chapter on GLYCOLYSIS.

General Summary of Information on the Pyridine-Nucleotide-Linked Anaerobic Dehydrogenases

The dehydrogenases of this group have in most cases been prepared in relatively purified form. Malic and glucose-6-phosphate dehydrogenase have been highly purified. The alcohol (yeast and animal tissues), lactic (animal tissues) and triosephosphate enzymes have been crystallized.

Lactic, malic and β-hydroxybutyric dehydrogenases are similar in many respects but are not identical. They catalyze the dehydrogenation of their respective hydroxy-acid substrates to keto acids. Ethyl alcohol is oxidized to acetaldehyde. Compounds such as glucose which contain an aldehyde group are catalytically oxidized to the corresponding acids.

The mechanism of hydrogen transport by these systems is complicated. The first acceptor in most cases is DPN, but isocitric, glucose-6-phosphate and glutamic (yeast and bacteria) dehydrogenases require TPN. Lactic and malic dehydrogenases have been shown not to be exclusively DPN-specific. Malic, β-hydroxybutyric, glutamic (animal), isocitric and glucose dehydrogenases react through the cytochrome system, but proof that this is true for

all the enzymes of this group is lacking. As a rule reactions catalyzed by dehydrogenases in which DPN acts as acceptor are easily reversible oxidation-reduction systems, whereas reactions in which TPN is the acceptor are not readily reversible.

Evidence obtained by the use of inhibitors indicates that the activities of isocitric and triosephosphate dehydrogenases are dependent on the presence of -SH groups; the activity of the glucose enzyme may be dependent on the presence of aldehyde groups in the molecule. Some physical constants for certain of the pyridine-nucleotide-linked dehydrogenases are given in Table 4.

TABLE IV

Physical Constants of Anaerobic
Pyridine-Nucleotide-Linked Dehydrogenases

Dehydrogenase	pH of Optimum Activity	Michaelis Constant K_m (in molarity of Substrate)	E'_o of system at T°C (in volts at pH 7.0)	Reference*
Malic	8.0-9.0	0.01	-0.169 37° -0.102 38°	Laki (1937) Lehmann and Hoff-Jorgensen (1939)
Lactic	9.3	0.01	-0.180 37°	Barron and Hastings (1934)
β-Hydroxy-butyric	7.3	0.005	-0.293 38°	Hoff-Jorgensen (1938)
Glutamic	7.3	0.0075	-0.03	Kalckar (1941)
Isocitric	7.0-7.5	$<1.25 \times 10^{-5}$	-0.30	Ochoa (1946)
Alcohol (yeast)	8.0	0.024 (alcohol) 0.0001 (aldehyde)	-0.163 25°	Kalckar (1941)
Glucose	6.0-8.0	0.07	-0.45	Kalckar (1941)
Glucose-6-phosphate			-0.43	Ochoa (1946)
Formic	5.5-6.0	0.007	-0.42	Woods (1936) Kalckar (1941)

*References apply only to the E'_o values.

UNCLASSIFIED DEHYDROGENASES

PYRUVIC DEHYDROGENASE

Thunberg (1920) showed that pyruvate in the presence of frog muscle causes reduction of methylene blue. Pyruvate is oxidized by various animal tissues (Peters et al. 1934, 1936; Krebs and Johnson, 1937; Lipmann 1937; Stumpf, Zarudnaya and Green, 1947) and by bacteria (Krebs, 1937; Barron and Lyman, 1939; Lipmann, 1939a; Stumpf, 1945).

Lipmann (1937) showed that methylene blue is reduced by pyruvate in the presence of brain tissue and the rate was maximum with 0.001 M pyruvate. The lactic acid bacterium, Lactobacillus delbrueckii, has been used extensively for the study of pyruvate oxidation (Lipmann, 1939a, 1941, 1944). An acetone dried preparation was obtained from the bacteria which oxidizes pyruvate to acetate and carbon dioxide. The enzyme has optimum activity over the pH range of 6.0 to 6.5.

Under anaerobic conditions a dismutation of pyruvate can occur with the production of lactate, acetate and carbon dioxide. Presumably this is a carrier-linked reaction between the pyruvic and lactic dehydrogenase systems. This reaction is represented as follows:

$$\underset{\text{Pyruvic acid}}{2CH_3COCOOH} + H_2O \longrightarrow \underset{\text{Acetic acid}}{CH_3COOH} + \underset{\text{Lactic acid}}{CH_3CHOHCOOH} + CO_2$$

The oxidation of pyruvate requires inorganic phosphate, cocarboxylase, flavin adenine dinucleotide (FAD) (Lipmann, 1939c), magnesium ions and the dehydrogenase. According to Lipmann the oxidation is represented as follows:

1. $\underset{\text{Pyruvic acid}}{CH_3COCOOH} + H_3PO_4 \longrightarrow \underset{\text{Pyruvic acid phosphate hydrate}}{CH_3\text{-}C(OH)OPO_3H_2\text{-}COOH}$

2. $\underset{\text{Pyruvic acid phosphate hydate}}{CH_3\text{-}C(OH)OPO_3H_2\text{-}COOH} + FAD \xrightarrow{\text{Dehydrogenase}} \underset{\text{Acetylphosphoric acid}}{CH_3\text{-}COOPO_3H_2} + CO_2 + \text{reduced FAD}$

3. $\underset{\text{Acetylphosphoric acid}}{2CH_3\text{-}COOPO_3H_2} + \text{Adenylic acid} \longrightarrow \underset{\text{Acetic acid}}{2CH_3COOH} + \text{Adenosine triphosphate}$

Flavin adenine dinucleotide is the hydrogen acceptor in reaction 2. The reduced dinucleotide is oxidized by oxygen under aerobic conditions. Methylene blue can take the place of oxygen as hydrogen acceptor. Under aerobic conditions hydrogen peroxide is formed and it in turn oxidizes pyruvic acid to acetic acid, carbon dioxide and water.

According to Lipmann (1941) pyruvate is dehydrogenated only in the presence of inorganic phosphate. This dehydrogenation results in the formation of energy-rich phosphate bonds which cause adenylic acid phosphorylation to give adenosine triphosphate (reaction 3). The primary oxidation product is known to be acetyl phosphate since synthetic acetyl phosphate transfers energy-rich phosphate to adenylic acid.

The dehydrogenase system of L. delbrueckii is similar to that of E. coli in that the oxidation is also coupled with adenylic acid phosphorylation. Stumpf (1945) studied pyruvic acid oxidation by Proteus vulgaris, but in this case inorganic phosphate is not required and acetyl phosphate is not an intermediate. Diphosphothiamine is required.

Animal Tissues as Sources of Enzyme

The pyruvic dehydrogenase of animal tissues has not been purified or obtained in soluble form. According to Krebs and Johnson (1937) animal tissues are capable of removing pyruvate by dismutation and by oxidation. The essential reaction in both cases may be oxidative decarboxylation to acetate and carbon dioxide. Under aerobic conditions oxygen is the final hydrogen acceptor, whereas pyruvate acts as the acceptor during dismutation.

Stumpf, Zarudnaya and Green (1947) described two enzymes from pigeon breast muscle which catalyze the oxidation of pyruvic and α-ketoglutaric acids. These enzymes require diphosphothiamine, but not inorganic phosphate, flavin adenine dinucleotide, adenosine triphosphate, cytochrome c or divalent metal ions. The enzymes activate their respective substrates to react with molecular oxygen, ferricyanide or methylene blue. The rate of oxidation depends on the partial pressure of oxygen.

Green, Loomis and Auerback (1948) reported the presence of a cyclophorase system in rabbit kidney which catalyzes the oxidation of pyruvate to carbon dioxide and water by way of the citric acid cycle. The activity of this system is greatest in rabbit kidney but it

is rather widely distributed in liver, heart, brain and muscle of the rabbit and other animals. Further information on the cyclophorase system is given in the chapter on THE CITRIC ACID CYCLE.

HYDROGENASE

Hydrogenase is the name given to the enzyme that enables an organism to use molecular hydrogen. It is of obvious significance for those autotrophic bacteria whose main source of energy is derived by oxidation of hydrogen (for example, the Hydrogenomonas, sometimes called the Knallgas bacteria) in which the oxidation is specifically linked to the fixation of carbon dioxide (Lee and Umbreit, 1940; Kluyver and Manten, 1942). The occurrence of the enzyme in typical heterotrophic species, as Escherichia coli, was established by Stephenson and Stickland (1931, 1932). Hydrogenase may be classified as a dehydrogenase since it activates the specific substrate, H_2, in a reversible manner (Green and Stickland, 1934):

$$H_2 \rightleftharpoons 2H \rightleftharpoons 2H^+ + 2 \text{ electrons.}$$

Stephenson (1947) notes that this reaction has the highest reduction potential of any known biological system (E_o' = -0.445 at pH 7.0 and 30° C.). It may, therefore, be important as an intracellular reducing agent serving to keep other enzymes in the reduced active state.

Because of this possible role in enzyme mechanisms its occurrence is of interest. Grohmann (1924) first showed that representatives of widely different groups of bacteria are capable of hydrogen oxidation. This has been confirmed by several workers for many bacteria and a few species of algae: E. coli and coli-acidilactici, and Vibrio Rubentschickii (Stephenson and Stickland, 1931, 1932), Clostridium Welchii (Woods, 1938), Aerobacter aerogenes and cloacae (Ordal and Halvorson, 1939), Micrococcus phosphoreus (Nakamura, 1940), Proteus vulgaris (Hoberman and Rittenberg, 1943), several Azotobacter species (Lee and Wilson, 1943), and the green algae Scenedesmus and Rhaphidium (Gaffron, 1940).

Gaffron (1940) indicates that the presence of hydrogenase does not determine the reaction in which hydrogen will take part, but rather the other activities of the cell determine the fate of the activated hydrogen. After activation the hydrogen can reduce oxygen, dyes such as methylene blue, nitrate, nitrite, hydroxylamine, amino acids, fumaric acid, inorganic sulfur compounds (sulfur, sulfite and thiosulfate), and one-carbon compounds, such as carbon dioxide, carbon monoxide, formaldehyde and methyl alcohol. The end-product of inorganic nitrogen is ammonia (Woods, 1938), of the inorganic sulfur compounds, sulfide, and of the one-carbon compounds, methane (Stephenson and Stickland, 1933). Stephenson (1947) reviewed the role of hydrogenase in many of these reactions including the part it plays in the reduction of amino acids. The reduction of compounds such as nitrate, nitrite, hydroxylamine and fumarate by E. coli in the presence of molecular hydrogen was studied recently by Lascelles and Still (1946, 1947).

The properties of hydrogenase have been studied in both resting suspensions of bacteria and in cell-free preparations (Bovarnick, 1941; Lee, Wilson and Wilson, 1942). The properties of the enzyme from different organisms appear to agree remarkably well, the outstanding one being sensitivity to oxidation. For this reason assay is best made in an atmosphere containing at least 0.4 atmosphere of hydrogen and less than 0.025 atmosphere of oxygen (Wilson, Lee and Wilson, 1942). The hydrogenase of Azotobacter shows optimum activity at pH 7.5 and the optimum temperature is 40° C.

Inhibitors of the cytochrome system, such as cyanide and carbon monoxide, are effective in decreasing the oxidation of hydrogen by intact cells of A. vinelandii (Wilson and Wilson, 1943). Sodium azide, hydroxylamine, iodoacetate and fluoride were found to inhibit the oxidation of hydrogen by Azotobacter cells in a quantitatively different manner than inhibitors such as cyanide. Concentrations of these compounds which prevent respiration do not affect the hydrogenase. Because of this these inhibitors can be used differentially to detect hydrogenase in cultures that have high endogenous respiration.

TABLE V

Unclassified Dehydrogenases and Some of Their Properties

Dehydrogenase	Source	Substrate	Carrier	Inhibitors	pH of optimum activity	References
α-Hydroxy-glutaric acid	Pig heart, rabbit & rat tissues	L-α-Hydroxy-glutaric acid	Pyocyanine & related phenazine dyes	Iodoacetate pyrophosphate & oxalate	8.0-8.5	Weil-Malherbe (1937b)
α-Keto-glutaric acid	Cat heart muscle	α-Keto-glutaric acid			7.5	Ochoa (1943)
Butyric acid	Guinea pig liver	Butyric acid and other acids	Cytochrome c	Fluoride, iodo-acetate, malonate and arsenate	7.7	Munoz and Leloir (1943)
Higher fatty acids	Rat liver & muscle	Palmitic acid and other acids	Methylene blue	Oxygen, cyanide, hydroxylamine & silver nitrate	7.0-8.0	Lang (1939), Lang & Mayer (1939), Burton (1948)
Phospho-hexonic acid	Yeast	6-Phospho-gluconic acid	TPN	Phosphate	6.3-7.5	Warburg, Christian & Griese (1935, 1937), Dickens (1938)
Pentose-phosphoric acid	Yeast	D-Ribose-5-phosphoric acid	TPN		6.4	Warburg, Christian & Griese (1935, 1937), Dickens (1938)
Hydro-enlyase*	Bacteria	Formic acid		Toluene, fluo-ride, cyanide & carbon monoxide	6.2-7.0	Stickland et al. (1929, 1932, 1933)
Sarcosine	Rat liver	Sarcosine	Cytochrome c		7.8	Bernheim & Bernheim (1942)

*The relation of the action of hydrogenlyase to that of hydrogenase is discussed in the section on HYDROGENASE.

Hoberman and Rittenberg (1943) showed that the exchange reaction between water and hydrogen when catalyzed by Proteus vulgaris is reversibly inhibited by oxygenation. Evidence was obtained which indicates that this inactivation is caused by the oxidation of a heavy metal component of the enzyme. On the basis of this it was suggested that hydrogenase is an iron porphyrin containing enzyme. The inhibition caused by cyanide and carbon monoxide appears to support this hypothesis.

Stephenson and Stickland (1932) and Stephenson (1937) indicated, on the basis of their relative distribution, that hydrogenase, hydrogenlyase and formic dehydrogenase are distinct enzymes. The latter enzyme activates the hydrogen of formic acid to react with an intracellular hydrogen carrier or with a dye such as methylene blue. Hydrogenlyase, on the other hand, was reported by Stickland (1929) to catalyze only the liberation of molecular hydrogen from formic acid. Ordal and Halvorson (1939) and Waring and Werkman (1944) obtained results which suggest that the production of carbon dioxide and hydrogen from formic acid by bacteria such as E. coli is due to the linkage of formic dehydrogenase to hydrogenase through an intermediate carrier containing iron. This makes unnecessary the postulation of formic hydrogenlyase as a distinct enzyme. Stephenson (1947) states that this hypothesis awaits further investigation and points out that the presence of iron in the growth medium conditions the formation of gas (H_2 + CO_2) producing enzymes.

The function of hydrogenase is evidently to serve as a means of utilizing molecular hydrogen as a hydrogen donator or more probably to liberate hydrogen by anaerobic bacteria by the reversal of the reaction cited above. Its role in aerobic organisms, such as the luminous bacteria, algae and the aerobic nitrogen fixer, Azotobacter, is not so evident. Nakamura (1940) suggests that hydrogenase takes part in the formation of luciferin by the light-producing bacterium, Micrococcus phosphoreus. Its presence in algae after a suitable period of adaptation enables these organisms to assimilate carbon dioxide by photoreduction in either dark or light instead of by photosynthesis (Gaffron, 1940, 1942). The role of hydrogenase in photosynthesis is discussed in the review by Stephenson (1947). Its occurrence in Azotobacter appears to be connected with the mechanism of nitrogen fixation (Wilson and Burris, 1947), but its exact role has not been established. It has not been detected in the nodules from leguminous plants fixing nitrogen or in pure cultures of the root nodule bacteria (Wilson, Burris and Coffee,,1943), although nitrogen fixation by the symbiotic system is inhibited by molecular hydrogen in a manner similar to that in Azotobacter.

A summary of information on additional UNCLASSIFIED DEHYDROGENASES is given in Table 5, on page 143.

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Chapter VII

CYTOCHROME SYSTEMS

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INTRODUCTION

The cytochrome systems discussed in this section will be considered in three main categories: 1) The cytochrome pigments, 2) The enzymes that reduce the cytochrome pigments, which will be referred to as cytochrome reductases, and 3) The enzyme or enzymes that oxidize the cytochrome pigment, which will be referred to as cytochrome oxidase.

According to present knowledge, which is based mainly on the work of Keilin and Hartree (1939) and of Ball (1938, 1942) electrons pass in order through cytochromes b, c, a and cytochrome oxidase to oxygen. Since only cytochrome c has been obtained in solution and in purified form our present knowledge is mainly in terms of the reduction and oxidation of this component. The cytochromes function biologically as electron transporting mechanisms, and are frequently referred to as "carriers". Whether they are involved in phosphate metabolism remains to be seen. Although there is reason to believe that they might be involved in oxidative phosphorylation, there is as yet no direct evidence on the point.

THE CYTOCHROME PIGMENTS

Earlier work on the cytochromes has been summarized by Keilin (1933). It is of interest to note that the pigments were discovered in 1886 by MacMunn who called the compounds myohematin. The substances were rediscovered by Keilin in 1925 and were found to consist of three components, on the basis of absorption bands seen in crude preparations or in tissue in situ. Keilin found the bands to be widely distributed in biological material. On the basis of the absorption spectra, new cytochrome components have been described whenever the absorption peaks do not fall at the characteristic wavelengths for the a, b, and c components. Thus a_1 and a_2 occur in certain bacteria lacking component a, according to Keilin and Hartree (1939), who also reported component a_3 in heart muscle preparations. The latter compound may be identical with cytochrome oxidase (see below).

Cytochrome b has been studied mainly in suspensions of muscle, where its reduced bands can be seen at 564, 530 and 432 mμ. Both b and b_2 are discussed below in connection with the reduction of c. According to Keilin and Hartree (1939) b can be oxidized by air without the intervention of c and its oxidase, and it is not clear as to what significance this fact may have from the quantitative standpoint.

Cytochrome c has been obtained in pure form in solution and is characterized by the 3 bands in the reduced state at 550, 521 and 415 mμ. A method of preparation was given by Theorell (1936). A more effective method of preparation was given by Keilin and Hartree in 1937 and this was further improved in 1945. A further purification by Theorell and Akesson (1939, 1941) increased the iron content from 0.34% to 0.43% and yielded a product with a molecular weight of 13,000 in comparison with the weight of 16,500 for the original product. Another method of preparing this compound was given by Keilin and Hartree in 1945, who found that the new material had the same catalytic activity per weight of iron as the former product. The spectrum of the reduced form was also the same per weight of iron. The significance of the protein removed by the new procedure is still obscure. A number of chemical studies on cytochrome c have been carried out by Theorell (1938, 1939, 1941) by Theorell and Akesson (1941) by Zeile and Meyer (1939 a, b, 1940) and by Drabkin (1941 a, b). The work by Theorell (1941) reaffirmed the previous view that cyanide could not form a complex with cytochrome c and is difficult to reconcile with the demonstration of a cyanide complex (see below) unless the spatial configuration proposed by Theorell does not stop but merely slows complex formation.

Theorell and Akesson concluded that cytochrome c from beef heart and horse heart were identical as to molecular weight, and nitrogen, iron, and sulfur content. They calculated that each molecule of protein contained 96 ± 1 amino acids, of which 21 or 22 were lysine residues, which probably represents the highest lysine content of any protein. They found 9 or 10 free α-amino groups, and suggested that the molecule contained this many polypeptide chains. With respect to the porphyrin portion of the molecule, Theorell's earlier

view that it was a dicysteine-porphyrin was supported by Zeile and Meyer although certain remaining uncertainties were discussed by Theorell and Akesson. Titration curves showed that the dissociation constants for the histidine-imidazole groups of the cytochrome molecule lie for the most part outside the normal titration region, and it was suggested that 2 of the 3 imidazole groups are hemochromogen-forming. This conclusion was supported further by magnetic and spectrophotometric measurements.

The determination of cytochrome c in tissues has been attempted by various authors. Stotz (1939) used manometric techniques based on the catalytic properties of cytochrome c: Potter and DuBois (1942) used a spectrophotometric method based on the change in optical density when cytochrome c was converted from the oxidized to the reduced form by specific enzymes. Rosenthal and Drabkin (1943) developed the spectrophotometric method further and modified the extraction procedure. None of these procedures would deal with quantities as small as were encountered in mouse epithelium and this fact led Carruthers (1943) to develop a polarigraphic method. Cytochrome c has been reported to be low in tumor tissues, paralleling the reduction in various oxidative enzymes (see Potter, 1944).

The enzymatic oxidation and reduction of cytochrome c is discussed below. It is perhaps worth noting that a large number of compounds that are more or less autoxidizable will also rapidly reduce cytochrome c, and can consequently be oxidized in a cytochrome c - cytochrome oxidase system. Keilin and Hartree (1939) have noted the properties of cytochrome c on the basis of their studies with muscle preparations, and reported that in the oxidized state it does not combine with KCN, H_2S or NaN_3. By considerably different experimental methods, Potter (1941) showed that cyanide forms with ferri-cytochrome c a complex that cannot be reduced by succinic dehydrogenase, and showed that a slight shift in the absorption maximum occurred. This work was confirmed by Horecker and Kornberg (1945) who studied the phenomenon in considerably greater detail.

Cytochrome c has been used intravenously for the treatment of various clinical conditions but the results are still controversial and the literature will not be summarized here.

CYTOCHROME REDUCTASES

The exact nature of the electron transport system between the anaerobic dehydrogenases and oxygen has never been fully worked out for animal tissues but the idea has gained wide acceptance that the pathway proceeds via cytochrome c and cytochrome oxidase. The problem of how the cytochrome c is reduced in animal tissues has never been resolved in terms of purified enzyme preparations. This is largely due to the fact that the enzymes involved are in insoluble particulate matter.

DPN-CYTOCHROME c REDUCTASE

The idea that a special enzyme was required for the transport of electrons between reduced coenzyme I (DPN) and the cytochromes was apparently arrived at independently at Stockholm (Adler, et al, 1937) and at Cambridge (Dewan and Green, 1937) after a period of intense activity in the study of the DPN-requiring dehydrogenases. At the time that the first edition of the present volume was written, dehydrogenases were identified in terms of methylene blue reduction, but during 1937-1938 a series of papers from the Stockholm and Cambridge laboratories clearly showed that DPN-requiring dehydrogenases cannot react directly with methylene blue, with oxygen, or with cytochrome c. The key to this new idea was the development of techniques that permitted the study of the dehydrogenases in the absence of the enzymes that linked them to methylene blue or to oxygen. The critical experiments involved the use of Meyerhof muscle extracts, which are incapable of catalysing the reactions between reduced DPN and methylene blue or oxygen but are capable of an active glycolysis. The following paragraph from Green, Needham and Dewan (1937), illustrates the situation: "Woods and Green (quoted by Green, 1936) failed to detect the presence of dehydrogenase systems in actively glycolysing muscle extracts. The tests applied for the presence of triose phosphate, lactate and α-glycerophosphate dehydrogenases were (1) reduction of methylene blue by the various substrates, and (2) reaction with molecular oxygen in the presence of carriers. These negative findings presented a paradoxical situation.

On the one hand, the evidence was complete that triosephosphate and glycerophosphate could be dehydrogenated to phosphoglycerate and that pyruvate could be hydrogenated to lactate. On the other hand, no evidence was forthcoming that the usual type of dehydrogenase system was responsible for these oxidoreductions."

It is now known that the above criteria are not adequate for the demonstration of dehydrogenase activity, but at that time the picture was less clear. The Cambridge group attempted to solve the dilemma by calling the enzymes "mutases" instead of dehydrogenases: "It was at once clear from this study by Dixon and Lutwak-Mann that enzymes of a type similar to the aldehyde mutase of liver must be responsible for the oxidoreductions observed in muscle extracts." But even while this paper was in press the old criteria for dehydrogenase activity were being outmoded, and "subsequent experimentation disclosed that dehydrogenases like mutases cannot catalyse the oxidation of reduced coenzyme by means of carriers".

Meanwhile the Stockholm group had taken the position that the oxidoreductions in muscle extracts were due to typical dehydrogenase systems, (Euler, Adler, Gunther and Hellstrom, 1936); (Adler, Euler, and Hughes, 1938). The availability of pure preparations of DPN and the development of ultraviolet spectrophotometry made it possible to show that DPN functioned by undergoing a cycle of reduction by triosephosphate and of oxidation by pyruvate. Furthermore, the enzymes catalyzing these reactions were in different fractions. Subsequently, pure lactic dehydrogenase and triosephosphate dehydrogenase were isolated from these extracts (see Chapter VI). The isolated dehydrogenases are not "typical" in the old sense, inasmuch as they do not reduce methylene blue, but by now it has become clear that this is no longer an adequate criterion of dehydrogenase activity, and that the latter is to be established solely in terms of dehydrogenation of the substrate. The activities formerly ascribed to "mutases" are therefore now known to be due to the coordinated function of 2 dehydrogenases.

With the advent of soluble dehydrogenase preparations that would react with neither oxygen nor methylene blue, it was possible to inquire into the nature of the former "dehydrogenase preparations" that had been able to take up oxygen or reduce methylene blue. These earlier preparations were in general not much more than suspensions of finely ground washed tissue. It was found that the suspensions included a soluble fraction that contained the "true" dehydrogenases and an insoluble fraction that was required for the reduction of methylene blue or the uptake of oxygen, but was not required for the reduction of DPN. It is the nature of the insoluble fraction that we are concerned with here. It was named "coenzyme factor" by Dewan and Green (1938) for the following reasons: "Strictly speaking the coenzyme factor is a dehydrogenase which catalyses the oxidation of its substrate, i.e. reduced coenzyme by means of various hydrogen acceptors. But to refer to the coenzyme factor as 'coenzyme dehydrogenase' would introduce confusion with 'coenzyme dehydrogenase systems'. It is preferable that the term dehydrogenase should be applied only to enzymes which are concerned in catalyzing the oxidation of metabolites." With the latter statement the present author is in agreement, but the ambiguity of "various hydrogen acceptors" in the above definition has never been clarified, and even today, in 1948, the immediate physiological oxidant for the coenzyme factor remains unknown. The same enzyme was named "diaphorase" by Euler and Hellstrom (1938). Both groups regarded the cytochromes as the physiological oxidants of the enzyme but the test system usually involved methylene blue reduction. Straub (1939) isolated a soluble flavo-protein from heart muscle and showed that the prosthetic group was flavinadenine dinucleotide, while Corran, Green and Straub (1939) tested the catalytic properties of the enzyme and concluded that it was identical with the coenzyme factor. But none of the above tests involved the reduction of any of the cytochromes. Dewan and Green (1938) showed that the insoluble enzyme would reduce cytochromes a and b (which it contained) but did not report whether it would reduce added cytochrome c. However, they concluded that "cytochrome c appears to take no part in the reaction between reduced coenzyme and oxygen," since added cytochrome c did not stimulate the rate of oxygen uptake. On the other hand we have shown that in fresh homogenates cytochrome c can be reduced by dihydro-DPN (Lockhart and Potter, 1941; Potter and Albaum, 1943), that cytochrome c is required for the aerobic oxidation of dihydro-DPN (Lockhart and Potter, 1941), and that cytochrome c stimulates the rate of oxygen uptake of DPN systems (Potter, 1946; 1947). The latter observations with fresh homogenates are of considerable importance since they differ from the earlier results of Ogston and Green

(1935) who found that in their preparations only the succinic and α-glycerophosphate dehydrogenases of animal tissues could react with cytochrome c. In this conclusion Ogston and Green failed to confirm the earlier results of Harrison on the stimulation of glucose dehydrogenase by cytochrome c. In a subsequent investigation, Hawthorne and Harrison (1939) reaffirmed the earlier position and showed that the conclusion of Dewan and Green (1938) regarding the non-participation of cytochrome c in the oxidative mechanism was based upon an experiment in which the result obtained depended upon the concentration of the insoluble preparation. With high amounts of the preparation, no cytochrome c effect was obtained, but at dilutions of 1:20 and 1:40 marked cytochrome c effects were demonstrated. It seems possible that the results of Dewan and Green may have been due to traces of cytochrome c in their insoluble preparation, while the earlier results of Ogston and Green were evidently due to the destruction of the coenzyme factor by the procedures used, since Potter and Lockhart (1941) showed that similar procedures when applied to fresh preparations of coenzyme factor or diaphorase destroyed the ability to reduce cytochrome c but not the ability to reduce methylene blue (cf. Eichel, B., and Wainio, W. W., J. Biol. Chem., 175, 155, (1948).

The present state of knowledge is thus still far from satisfactory. In the reduction of cytochrome c by reduced DPN in animal tissues it is not known whether the reaction is catalyzed by a single enzyme, or whether it is reduced by the joint action of two systems, for example, diaphorase (the soluble form) plus cytochrome b (insoluble particles). In any event, it is certain that the reaction takes place, and the enzyme that catalyzes it can be most specifically designated as DPN-cytochrome c reductase (cf. Potter, 1946). The relation between diaphorase and this enzyme must remain obscure until further experiments have been done. Straub (1942) obtained a preparation (the SC-factor) that would mediate the electron transfer between succinic dehydrogenase and cytochrome c, but neither the SC-factor nor diaphorase or a combination of the two would mediate the transfer from reduced DPN to cytochrome c. An alternative pathway from reduced DPN to oxygen by-passing cytochrome c (Dewan and Green, 1938) is conceivable but cannot be regarded as established.

On the other hand, a soluble DPN-cytochrome c reductase has been prepared from yeast (Altschul, Persky and Hogness, 1941; Persky, 1942) but attempts at further purification have not been reported (see Heppel, L. A., Fed. Proc., 8, 205 (1949).

A method for the manometric determination of DPN cytochrome reductase in tissue homogenates has been proposed by Potter (1946), and a spectrophotometric method was outlined by Potter and Albaum (1943).

TPN-CYTOCHROME c REDUCTASE

This enzyme was obtained in soluble form from yeast and was brought to a stage that represented 98 per cent purity (Haas, Horecker, and Hogness, 1939, 1940; Haas, Harrer and Hogness, 1942). The enzyme specifically catalyzes the reaction between reduced TPN (triphosphopyridine nucleotide, coenzyme II) and cytochrome c. It was originally called cytochrome c reductase but we have added the prefix TPN to distinguish it from the DPN enzyme (Potter, 1949). (See Horecker, B. L., Fed. Proc., 8, 207, (1949).)

The earlier preparation appeared to contain not more than 3% of a hemin protein thought to be identical with cytochrome b_2 but the final preparation was free from this substrate. Thus it can be concluded that in at least one instance the path between a reduced coenzyme and cytochrome c has been shown to be catalyzed by a single enzyme, obtainable in soluble form and free from the other cytochrome pigments.

The enzyme was shown to be a flavoprotein, having alloxazine mononucleotide (riboflavin phosphate) as the prosthetic group. The dissociation constant was 1×10^{-9} mole/liter, a value 1/60 that of the "old" yellow enzyme of Warburg and Christian, which has the same prosthetic group. While the "old" enzyme would react with either oxygen or cytochrome, depending on their relative concentration, (Theorell, 1936) the new enzyme reacted with cytochrome c much more readily than with oxygen.

SUCCINIC-CYTOCHROME c REDUCTASE

For many years it had been assumed that succinic dehydrogenase reacts directly with cytochrome c but in 1939 Hopkins, Morgan and Lutwak-Mann obtained preparations that would reduce methylene blue but would not take up oxygen in the presence of cytochrome c although cytochrome oxidase was present. Similar results were also obtained by Stern and Melnick (1939) and Keilin and Hartree (1940). It remained for Straub (1942) to reconstruct the system however. Like the earlier workers he obtained a preparation that would reduce methylene blue but not cytochrome c, but in addition he obtained a fraction that would link the succinic dehydrogenase to cytochrome c, and this was called the "SC-factor". The work has not as yet been repeated but it is strikingly similar to the earlier work with the coenzyme factor and diaphorase, in that preparations could be obtained that would reduce methylene blue but not cytochrome c. In the DPN reaction, however, no cytochrome reducing factor has been obtained from animal tissues, and Straub found that this SC-factor was ineffective in a DPN system.

Slater (1948) has obtained evidence for an additional factor in the succinoxidase system between cytochrome b and cytochrome c, which is inactivated by 2,3 dimercaptopropanol ("BAL"), and cannot be replaced by Straub's SC-factor. Along similar lines, Stoppani (1947) fractionated rabbit or ox liver to obtain a soluble factor which activated an incomplete succinoxidase system that could be shown to contain succinic dehydrogenase and cytochrome oxidase. Also working with the succinoxidase system Keilin and Hartree (1947b) obtained a marked inhibition with hematin, and showed that the inhibition could be prevented by the presence of denatured globin. (See Keilin, D., and Hartree, E. F., Biochem. J., 44, 205, (1949).)

CYTOCHROMES b AND b_2

The oxidation reduction potential of cytochrome b was found by Ball (1938) to be about -0.04 v. while that of c and a were +0.27 and +0.29 respectively. It is apparent that cytochrome b, which is associated with insoluble cell-components, must be considered as a possible essential factor in the reduction of cytochrome c. However, the isolation of the TPN-cytochrome reductase from yeast (see above) and the fact that Straub's SC-factor does not function with diaphorase suggests that if cytochrome b is concerned in the reduction of cytochrome c, it is not the sole mechanism for such a reaction. The experiments of Ball certainly indicate that cytochromes b and c are in equilibrium in the cell. The work of Keilin and Hartree (1939) also strongly suggests that b is involved in the reduction of c. In addition to b, a new cytochrome (b_2), with an absorption maximum near that of b, has been found in yeast (Bach, Dixon, and Keilin, 1942; Bach, Dixon and Zerfas, 1946). The substance appeared to be identical to or closely associated with the yeast lactic dehydrogenase that does not require DPN. Crude preparations would reduce cytochrome c while purified preparations were more active with methylene blue. The substance was extremely labile.

CYTOCHROME OXIDASE

This name has been given to the enzyme that specifically oxidizes reduced cytochrome c. (Keilin and Hartree, 1938). It was earlier known as indophenol oxidase, but Keilin and Hartree showed that all of the compounds that had been thought to be oxidized by indophenol oxidase were in fact oxidized by cytochrome c, which was then oxidized by the oxidase. The new name is therefore the one to be employed. Whether cytochrome oxidase is identical with cytochrome a_3, which was discovered by Keilin and Hartree (1939) on the basis of its absorption bands in heart muscle preparations remains to be seen. Keilin and Hartree noted many similar properties that tended to identify a_3 as cytochrome oxidase but they were unable to reduce it with reduced cytochrome c, while cytochrome oxidase must obviously possess this property, although the spectroscopic experiment may have technical difficulties not yet overcome.

Haas (1943, 1944) reported the preparation of a "soluble" cytochrome oxidase and its separation into two components but these results were criticized by Keilin and Hartree (1947a) who considered that the centrifugal force of 10,000 g employed by Haas yielded a supernatant fluid containing colloidal particles rather than a true solution. They also

doubted that his separation of cytochrome oxidase into 2 components was valid inasmuch as they were able to repeat his results by substituting for his Fraction II with denatured globin or with plasma proteins. Wainio et al (1948) have reported a "soluble" cytochrome oxidase preparation based upon the extraction of the tissue particles with sodium desoxycholate as in the work of Hopkins et al (1939) and Straub (1942).

Numerous methods for the determination of cytochrome oxidase have been proposed. For tissue assays, the water homogenate is probably the best starting material, and the manometric technique is undoubtedly to be preferred. Schneider and Potter (1943) reduced cytochrome c by means of ascorbic acid and described conditions for the assay.

A cytochrome c peroxidase has been obtained from yeast (Altschul, Abrams and Hogness, 1939, 1940; Abrams, Altschul and Hogness, 1942). It catalyzes the reaction between reduced cytochrome c and H_2O_2. Whether it occurs in animal tissues is unknown as is its functional significance in yeast.

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Chapter VIII

OXIDASES, PEROXIDASES AND CATALASE

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OXIDASES, PEROXIDASES AND CATALASE

The oxidases, catalase and peroxidase share the property of being metalloprotein enzymes. Their catalytic activity is dependent upon the presence of a copper or iron bearing moiety bound to their protein. Catalase and peroxidase contain porphyrin bound iron, whereas tyrosinase, laccase, ascorbic acid oxidase and perhaps cytochrome oxidase* contain copper.

Molecular oxygen serves as the electron acceptor for the oxidases. The oxidases activate O_2; subsequent to its activation the O_2 can accept hydrogen (electrons) to form water. No detectable hydrogen peroxide is produced in the reaction. Thus, in the stepwise transfer of electrons, which occurs in the oxidation of a substrate, the final step resulting in the reduction of O_2 is mediated by the oxidases.

Catalase performs the highly specialized function of catalyzing the decomposition of hydrogen peroxide to water and O_2. Although difficult to detect, hydrogen peroxide is pictured as a common end product of biological oxidations; the widespread occurrence of catalase assures that it will not accumulate in tissues to toxic concentrations. Catalase may also serve as a peroxidase, and current concepts stress that this is likely a more important physiological role for the enzyme than is the direct decomposition of hydrogen peroxide.

Peroxidases catalyze the oxidation of substrates by hydrogen peroxide. Although these iron protein enzymes are widely distributed in plants, their quantitative importance in overall respiration is ill-defined.

OXIDASES

The term oxidase has been used loosely and often has been applied to certain dehydrogenases. In this chapter we shall restrict the term to the metalloprotein enzymes which activate O_2 and in its reduction form H_2O but not H_2O_2. The enzymes included under such a definition are tyrosinase, catecholase, laccase, dopa oxidase, ascorbic acid oxidase, and cytochrome oxidase. Other enzymes under the tyrosinase group may eventually prove to be distinct entities but their broad substrate specificity prompts their consideration under one heading at present.

TYROSINASE

Bourquelot and Bertrand (1895) obtained a crystalline material from the mushroom, Russula nigricans, which was oxidized by the mushroom juice to a red and finally to a black substance. Bertrand (1896) demonstrated that the crystalline substance was tyrosine. As it was not oxidized by laccase and the mushroom enzyme did not oxidize hydroquinone (hydroquinone is oxidized by laccase), Bertrand considered the new enzyme distinct from laccase and designated it tyrosinase. Tyrosinase has also been termed polyphenol oxidase, catechol oxidase, phenolase and potato oxidase. Other identical or closely related enzymes have carried additional names. Whereas most enzymes show a high degree of substrate specificity, tyrosinase not only catalyzes the oxidation of a variety of polyphenols but also a number of monophenols. The term tyrosinase has been preferred by Nelson and Dawson (1944) on the basis of priority and because it does not emphasize the activity on polyphenols to the exclusion of the monophenols. The broad specificity of the enzyme doubtless accounts for its plethora of names.

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*Cytochrome oxidase is not considered here but is discussed in Chapter VII.

The enzyme is widely distributed and appears to be of special significance in the plant kingdom. In many plants tyrosinase is by far the most active respiratory enzyme demonstrable; it may occur in both the tops and the underground parts of plants. Its distribution and importance seem to be more restricted in animals, but the enzyme has been found in many invertebrates and has been reported to be present in the skin and in melanomas of higher animals.

Tyrosinase has been studied extensively and an enormous and confusing literature regarding it has accumulated. Within recent years the outstanding work of Kubowitz, of Keilin and Mann, and of Nelson and coworkers has aided greatly in clarifying our knowledge of the enzyme.

Kubowitz (1937, 1938) succeeded in isolating a highly purified tyrosinase from potatoes. The peels of potatoes were employed for the preparation and their aqueous extract was subjected to a series of treatments with acetone, ammonium sulfate, silver acetate and aluminum hydroxide (Kubowitz, 1937, gives details). Analysis of the yellow-colored enzyme solution established that it was a copper containing protein. Kubowitz considered that his best preparations containing 0.20% copper were the pure enzyme.

Keilin and Mann (1938) prepared polyphenol oxidase from the mushroom, Psalliota campestris. A water extract of the minced mushrooms was successively precipitated with ammonium sulfate, dialyzed, precipitated with lead acetate, adsorbed on tricalcium phosphate gel, fractionally precipitated with acetone plus ead acetate, readsorbed on tricalcium phosphate gel and finally precipitated with acetone. The purified preparation from mushrooms carried 0.30% copper. Keilin and Mann as well as Kubowitz purified their enzymes to constant copper contents, so it is not unlikely that tyrosinase from potato and from mushroom do have different levels of copper. If the weight of the protein constituent of the enzyme were distinctly different in the two cases the percentage of copper should reflect this. The alternate explanation, that the enzymes were not entirely pure, remains possible. Keilin and Mann did not claim that their enzyme was pure but believed this probable.

The details of another method for preparing highly purified tyrosinase from mushrooms have been described recently by Mallette, et al. (1948).

The activity of tyrosinase was measured in different manners by Kubowitz and by Keilin and Mann, so it is not valid to compare their Q_{O_2} data directly. The Q_{O_2} on catechol of the preparation from potatoes was about 30,000, whereas the mushroom preparation had a Q_{O_2} of 1,170,000 on catechol.

Kubowitz (1937) demonstrated that the activity of his tyrosinase preparations was proportional to their copper content. It was the experience of Keilin and Mann that this proportionality was not apparent until the preparations were reasonably well purified; at earlier steps in the separation contaminating copper confused the picture. This observation of Kubowitz (1937) was of particular interest, as it was the first proof that copper may serve as a constituent of oxidative enzymes. In the presence of HCN tyrosinase splits into copper and a protein; these can be separated by dialysis. By splitting copper from the metalloprotein enzyme, Kubowitz could demonstrate that the copper alone or the protein alone was inactive. The addition of one "equivalent" of copper to the protein restored 56% of the original enzymatic activity and 10 equivalents of copper restored 98% of the activity. Other metals, including iron, cobalt, nickel, manganese and zinc did not restore activity to the enzyme. Copper thus appeared to be unique in its ability to function in tyrosinase.

Tyrosinase is less specific than many enzymes, for it will oxidize a considerable variety of phenolic substrates. The specificity is confined to monophenols and to polyphenols with the second hydroxyl ortho to the first. The oxidation of the monophenols apparently requires their initial conversion to an o-dihydric phenol. From this point the o-dihydric phenols are oxidized by the removal of hydrogen (electrons) to their corresponding o-quinones. Table 1 lists substrates attacked by tyrosinase.

TABLE I

Substrates Reported to be Oxidized by Tyrosinase

Monohydric Phenols	Polyhydric Phenols	Not Oxidized
tyrosine	catechol	hydroquinone
phenol	homocatechol	resorcinol*
p-cresol	urushiol	ascorbic acid*
guaiacol	pyrogallol	
tyramine	protocatechuic acid	
p-hydroxyphenylethylamine	chlorogenic acid	
p-hydroxyphenylmethylamine	caffeic acid	
p-hydroxyphenylamine	3,4-dihydroxylphenyl-L-alanine	
p-hydroxyphenylpropionic acid	adrenaline	
p-hydroxyphenylacetic acid	4-methylcatechol	
	4-(N-methyl-2'-aminoethyl)-catechol	
	4,5-dimethylcatechol	
	4-tert-butylcatechol	
	3-methylcatechol	

*Oxidized in the presence of catechol.

The data of Table I emphasize that the specificity of tyrosinase is concerned primarily with the hydroxyl groups; the presence of complex side-chains in urushiol and chlorogenic acid does not prevent their being oxidized. Cushing (1948) has tested the ability of mushroom tyrosinase to oxidize a great variety of substituted compounds, many of which are not listed in Table I, and has given a detailed analysis of the properties of substances active and inactive as substrates.

For test purposes p-cresol and catechol are most frequently used to follow the monophenolase and polyphenolase activities of the enzyme. Tyrosine is oxidized much less rapidly than is catechol. Kubowitz (1938) observed that catechol was oxidized 124 times as rapidly as tyrosine but calculated that diffusion limited the catechol oxidation and that the true rate of catechol oxidation was about 1100 times that of tyrosine.

It is difficult to establish the true rate of catechol oxidation, as the rate decreases with time. Kubowitz has attempted to overcome this by reconstructing a system which includes a very low concentration of catechol serving as a carrier and in which hexosemonophosphate (plus its dehydrogenase and triphosphopyridine nucleotide) acts as the electron donor to keep the o-quinone reduced. Another method for achieving a reasonably constant rate of oxidation by tyrosinase is to choose a substrate, such as protocatechuic acid, whose inactivation of the enzyme is very slow. Miller, et al. (1944) described a "chronometric method" for measuring the initial rate of catechol oxidation. It depends upon "the measurement of the time necessary for a given quantity of the enzyme to produce o-benzoquinone in the catechol-enzyme system just in excess of that necessary to oxidize a small amount of ascorbic acid also present in the system". The reaction mixture is continuously sampled into an acidified starch iodide indicator solution, and the appearance of o-benzoquinone at the end point time is evidenced by production of a blue color.

It has been suggested recently (Sizer, 1946) that tyrosinase can oxidize tyrosine present in an intact protein molecule. When tyrosinase was added to the intact proteins trypsin, pepsin, chymotrypsin, casein or insulin an uptake of oxygen was observed. Edman (1947) has challenged this work and has attributed the uptake of oxygen to the oxidation of free tyrosine present with the proteins initially and arising by autolysis during the experiment. Sizer (1947) repeated the experiments with carefully purified proteins from which free tyrosine and peptides had been removed. Although some autolysis occurred during the measurements, oxidation of the autolytic products was insufficient to account for

the observed oxygen consumption. The experimental results strongly indicated that tyrosyl groups in the intact proteins were oxidized by tyrosinase.

Tyrosinase is a reasonably heat stable enzyme, for Kubowitz used differential heat denaturation to remove impurities from his preparation. He heated a solution of the enzyme in phosphate buffer at pH 7.4 for 4 minutes at 59-60° C. and then cooled quickly and centrifuged off the denatured protein; this procedure did not reduce the enzymatic activity very much. The stability of tyrosinase generally increases with purification of the enzyme. Concentrated, purified preparations of tyrosinase remain active for months at refrigerator temperatures, but they are inactivated rapidly upon dilution. Nelson and Dawson (1944) have observed that the monophenolase activity of the enzyme is less stable than the polyphenolase activity.

The very low energy of activation of tyrosinase is of special interest. Whereas other enzymes exhibit an energy of activation in the range 5,000 to 20,000 cal., tyrosinase has an energy of activation of 2,700 cal. (Gould, 1939). Other oxidases with values as low as 5,200 cal. have been reported, but the dehydrogenases have uniformly high energies of activation. The value of 2,700 cal. was observed for tyrosinase preparations from potato, mushroom and the mealworm (Tenebrio molitor) acting either on catechol or p-cresol. This suggests a close similarity of tyrosinases from various sources, and that a single enzyme is involved in both the mono- and polyhydric phenol activity of tyrosinase.

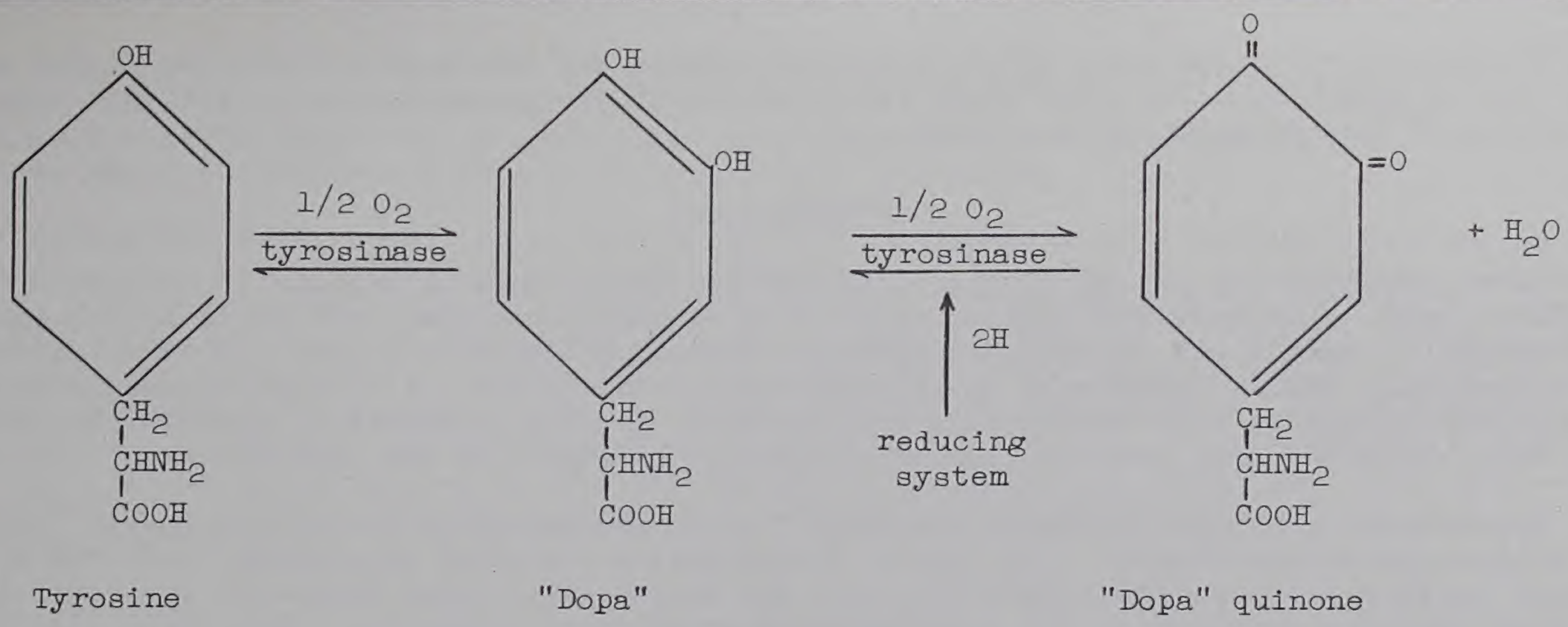
Tyrosinase is inhibited by CO, and the CO complex formed is not decomposed by light. Tyrosinase is inhibited also by low concentrations of cyanide, azide and sulfide as would be anticipated from its metalloprotein nature. In addition its action is blocked by 4-nitrocatechol (Gregg and Nelson, 1940) and by such copper inhibitors as diethyldithiocarbamate, salicylaldoxime, phenylthiourea, alpha-naphthylthiourea, allylthiourea, and thiourea (DuBois and Erway, 1946).

There is rather general agreement that the oxidation of monophenols by tyrosinase involves an initial addition of oxygen to form an hydroxyl group ortho to the one already present. There is frequently a lag in the rate of oxidation of monophenols, whereas this is not observed with the polyphenols. The lag in all likelihood corresponds to the time required for priming the reaction by the formation of a reservoir of a polyphenolic compound. This view is substantiated by the shortening of the lag period upon the addition of a small quantity of a polyphenol to the monophenolic substrate. After this initial conversion the oxidation of the mono- and polyphenols should follow a common pathway.

Tyrosinase catalyzes the oxidation of the orthodihydric phenols to their corresponding quinones. The subsequent oxidations which the o-quinones undergo give rise to the melanin pigments. Raper's (1932) studies on the formation of melanin indicate that tyrosine is converted to 3,4-dihydroxyphenyl-L-alanine (dopa) which in turn is oxidized to dopa quinone. Further oxidation gives rise to 5,6-dihydroxydihydroindole-2-carboxylic acid, its quinone (hallochrome), and finally melanin.

Of more general interest in biological oxidations than melanin formation is the function of the tyrosinase substrates in the capacity of electron carriers. In the presence of the proper reducing agents in the tissue the polyphenol is alternately oxidized to the quinone and reduced back to the polyphenol and is not oxidized further to melanin. In such a reaction the polyphenol serves the function of a carrier mediating the transfer of electrons from some substrate to O_2. Tyrosinase serves as the terminal oxidase for the overall oxidation system concerned.

Robinson and Nelson (1944) secured evidence to indicate that 3,4-dihydroxyphenyl-L-alanine (dopa) and its quinone may serve as the polyphenol-quinone pair for the tyrosinase system in intact plant systems. Tyrosine serves as a reservoir of dopa; as mentioned, the initial step in the oxidation of a monophenol is its conversion to the corresponding o-dihydric phenol.



Tyrosine "Dopa" "Dopa" quinone

One of the particularly interesting unsolved problems concerning tyrosinase is the nature of the systems which reduce the quinone to the polyphenol. While tyrosinase maintains the shuttle to O_2, these systems serve as electron donors. Experimentally ascorbic acid can function as the reducing agent. Robinson and Nelson (1944) have shown that as long as reduced ascorbic acid is present in a potato or mushroom tyrosinase preparation the solution remains colorless. After oxygen sufficient for oxidation of the ascorbic acid added has been taken up, the tyrosine and dopa present are oxidized further through the red intermediate to the black melanin pigments.

Boswell (1945) has reported that in intact potato tissue amino acids may serve for the reduction of dopa quinone. His evidence, however, is incomplete and it remains an open question what compounds are actually functional as reducing agents in the tyrosinase system.

Of what physiological importance is tyrosinase? Its wide distribution in the plant kingdom and its high activity suggest that it may serve as a major respiratory system in plants. The concept that it is functional only in injured plants has largely been displaced by the idea that it accounts for a considerable share of the plant's respiration under normal conditions. Boswell and Whiting (1938) observed that the addition of catechol to potato tuber slices was followed by a rapid uptake of oxygen, but that after a period the rate of respiration dropped until it was finally only about a third the rate established before the addition of catechol. They reasoned that the tyrosinase had been inactivated and that the residual third of the initial respiration rate represented non-tyrosinase respiration and the other two-thirds of the normal initial respiration could be attributed to the action of tyrosinase. Baker and Nelson's (1943) demonstration that the further addition of catechol resulted in another increase in the rate of oxygen uptake made them question such an interpretation. By testing the sensitivity of potato tuber slices to 4-nitrocatechol and other inhibitors they concluded that about 85% of the total respiration went through the polyphenol oxidase system. Although they disagreed with the experimental basis of the argument of Boswell and Whiting, Baker and Nelson confirmed the opinion that tyrosinase constitutes the major respiratory system of potato tubers and perhaps of many plants. Levy and Schade (1948) have recently challenged the generally accepted view that tyrosinase is the chief terminal oxidase in potato tubers and have suggested that cytochrome oxidase and some unknown system are the principle agents in potato respiration. However, they were unable to demonstrate cytochrome c in potatoes, and they did not discuss the implications of the fact that tyrosinase has actually been isolated from potato tubers (Kubowitz, 1937).

The role of tyrosinase in the invertebrates is less clear than in plants. The mealworm is the most commonly used source of invertebrate tyrosinase, and it yields an enzyme with characteristics apparently the same as those of the plant enzyme. Bodine and coworkers (1944) have reported the presence of a protyrosinase in the diapause egg of the grasshopper. This protyrosinase can be activated by heat (55-70° C.), or by treatment with sodium dioctylsulfosuccinate (aerosol OT), urea, sodium dodecylsulfate or sodium oleate.

Tyrosinase, although much less widely distributed and of lower activity in higher animals than in plants and invertebrates, may function in the pigmentation of skin and the production of the pigments of melanomas.

CATECHOLASE

Sweet potatoes contain an oxidase somewhat different from tyrosinase or laccase. Graubard (1939) expressed the opinion that it is a distinct enzyme, for it is active only upon catechol, pyrogallol, 3,4-dihydroxyphenyl-L-alanine and adrenaline. It oxidizes fewer substrates than either tyrosinase or laccase; unlike tyrosinase it attacks no monophenols and unlike laccase it does not oxidize hydroquinone, p-phenylenediamine, guaiacol or vanillin. All three enzymes, however, appear to oxidize catechol in the same manner.

The enzyme from sweet potatoes has also been investigated by Walter and Nelson (1946) and Rudkin and Nelson (1947). The latter investigators isolated chlorogenic acid and a closely related compound from sweet potatoes and showed that these compounds can serve as electron carriers adjacent to the terminal oxidase of sweet potatoes. They imply that the enzyme is distinct from potato and mushroom tyrosinase. As yet catecholase has been studied but little, and final decision as to its separate identity should perhaps be reserved for the future.

LACCASE

Yoshida (1883) described a heat labile substance in the latex of the Japanese lacquer tree to whose action he attributed the darkening of the latex upon standing. The enzyme responsible for the darkening was investigated by Bertrand (1894) and given the name laccase; his source material was the Indo-Chinese lacquer tree, Rhus succedanea. The enzyme oxidized a number of polyphenols.

Keilin and Mann (1939) purified laccase from Rhus succedanea by a method very similar to that they employed with mushroom tyrosinase. Their purified enzyme oxidized polyphenols and diamines including p-phenylenediamine and catechol. Unlike tyrosinase, laccase was inactive on monophenols such as p-cresol and tyrosine. The enzyme preparation had a deep blue color, was devoid of Fe or Mn but had 0.154% copper; later preparations (Keilin and Mann, 1940) had up to 0.24% copper. Laccase was inhibited by KCN, H_2S, NaN_3, diethyldithiocarbamate but not by CO. The activity of the preparation was proportional to its copper content, so the enzyme, like tyrosinase, appeared to be a copper protein. Keilin and Mann calculated that a pure preparation would likely have 0.34% copper. The chief impurity in the enzyme was a polysaccharide.

Keilin and Mann (1940) prepared laccase from Japanese and Burmese lacquer trees and found that the copper protein enzyme was again accompanied by a blue color. They were unable to prepare an active enzyme devoid of the blue color, but could get the blue pigment separated from the enzyme. The colored substance contained no copper nor protein but was regularly mixed with a carbohydrate. It could be reduced to a leuco-form. It is quite possible that the blue substance functions in the action of the enzyme, although this point is not established.

The possibility was investigated by Keilin and Mann (1940) that the absence of some material from the laccase preparation was responsible for its inability to oxidize monophenols. However, the addition of crude extracts of potato or mushroom did not activate the laccase for the monophenol p-cresol. Instead the mushroom and potato juices lost their ability to oxidize monophenols. As this inhibitory property was not affected by dialysis, was not exhibited by pigment preparations devoid of laccase, and was destroyed by boiling the laccase, it was concluded that laccase itself acted as the inhibitor of monophenol oxidation.

Gregg and Miller (1940) prepared laccase from the wild mushroom Russula foetens. When hydroquinone was employed as the substrate other plant oxidases did not interfere with the measurements. The starting material for the preparations was limited, so that purification sufficient to establish that the enzyme itself contained copper could not be achieved. The enzyme was remarkably stable to pH changes; it was not inactivated between

pH 2 and 9.5, whereas tyrosinase was destroyed quickly at pH 2. Hydroquinone, p-phenylenediamine, chlorohydroquinone, catechol and dimethylcatechol were oxidized. Though the oxidation of catechol appeared to follow the same pathway with laccase as with tyrosinase, laccase was not inactivated in the process as was tyrosinase.

Keilin and Mann (1939) listed the properties in which laccase differs from the tyrosinase (polyphenol oxidase) of cultivated mushrooms; (1) laccase is blue whereas tyrosinase is yellowish or colorless; (2) laccase oxidizes p-phenylenediamine more rapidly than catechol, whereas tyrosinase scarcely attacks p-phenylenediamine; (3) laccase, when pure, does not oxidize p-cresol at all whereas tyrosinase does; (4) crude laccase preparations oxidize ascorbic acid directly, whereas crude or pure tyrosinase oxidizes ascorbic acid only through a carrier like catechol; (5) laccase is not poisoned by CO, whereas tyrosinase shows a strong inhibition by CO; (6) the activity of laccase is of the same order as that of potato oxidase (Kubowitz, 1938) but only about a twentieth that of tyrosinase from cultivated mushrooms.

DOPA OXIDASE

Dopa, 3,4-dihydroxyphenyl-L-alanine, is oxidized by tyrosinase, catecholase, laccase, peroxidase and cytochrome oxidase plus cytochrome c (Graubard, 1939). In addition there is evidence that it is oxidized by a very specific enzyme, dopa oxidase, which occurs in certain animal tissues.

Bloch (1917) treated frozen sections of skin with dopa and within 24 hours noted that a dark pigment was deposited in the cytoplasm of the melanoblasts. He attributed the action to an enzyme because it was inhibited by heat or by cyanide. No reaction was observed with tyrosine and other substrates tested, so Bloch considered that a highly specific enzyme was involved and termed it dopa oxidase.

Figge (1940) suggested that melanin formation could be accounted for in certain animal tissues by the action of tyrosinase on tyrosine, but his supporting evidence was derived chiefly from analogy with plant tissues.

The most convincing evidence for the occurrence of a dopa oxidase and for a tyrosinase in mammals is that presented by Hogeboom and Adams (1942). They studied a mouse melanoma; this occurs as a soft, jet black encapsulated nodule which likely has its origin in skin melanoblast cells. The tumor was ground and its activity tested on phenylalanine, tyrosine and dopa. The phenylalanine was not oxidized but the other two substrates supported oxygen uptake and yielded a melanin. The tumor brei was centrifuged and the enzyme remaining in the supernatant was fractionated by ammonium sulfate precipitation. This fractionation yielded (a) dopa oxidase without activity on tyrosine, and (b) a fraction with an active tyrosinase but lowered activity on dopa.

The dopa oxidase oxidized 3,4-dihydroxyphenyl-L-alanine with an uptake of approximately 4 atoms of oxygen per molecule to yield melanin. The enzyme was heat labile and completely inhibited by 0.001 M. cyanide. The pH optimum was between 7 and 8. An outstanding property of the enzyme was its high specificity, for it did not oxidize tyrosine, hydroquinone, p-benzylhydroquinone or p-cresol; catechol was oxidized at a seventh and adrenaline at an eighth the rate of dopa.

The tyrosinase from mouse melanoblasts was somewhat different in substrate specificity from plant tyrosinases; it did not oxidize phenol or p-cresol. Monobenzylhydroquinone completely inhibited the tyrosinase, whereas it is oxidized by mushroom tyrosinase to a red pigment. The cyanide sensitivity of the animal tyrosinase and dopa oxidase is evidence that they are metalloprotein enzymes similar to the purified plant tyrosinases.

Hermann and Boss (1945) described an enzyme occurring in the ciliary bodies of cattle eyes. They concentrated the enzyme from ground ciliary bodies by differential centrifugation and tested its action on catechol, dopa and hydroquinone. In the absence of added cytochrome c, only dopa was oxidized, but all three substrates were oxidized in its presence. These observations again demonstrate the occurrence of a specific dopa oxidase in the tissues of higher animals.

ASCORBIC ACID OXIDASE

Ascorbic acid oxidase is a copper protein enzyme which is widely distributed among plants. There is no clear demonstration of its occurrence in animals. Szent-Györgyi (1928) observed that his "hexuronic acid" (ascorbic acid), isolated from adrenal cortex or orange juice, was oxidized by peroxidase plus a phenol and hydrogen peroxide. He postulated that a quinone formed from the phenol was reduced back to the phenol by ascorbic acid, yielding dehydroascorbic acid as the oxidized product. Later, Szent-Györgyi (1930, 1931) discovered that in addition to this non-specific route of ascorbic acid oxidation the acid could also be oxidized by a specific enzyme, "hexoxidase", which he found in cabbage leaves. Enzymes with comparable properties were also studied early by Zilva (1934), Tauber and Kleiner (1935), Hopkins and Morgan (1936) and by Srinivasan (1936). Tauber and Kleiner first applied the name ascorbic acid oxidase to the enzyme.

It is clear that ascorbic acid may be oxidized by functioning in the reduction of quinones formed by the action of various phenol oxidases and peroxidases. However, only its oxidation by the specific ascorbic acid oxidase will concern us in the following discussion.

The occurrence of a true ascorbic acid oxidase was rather well established by the workers listed above. However, Barron, et al. (1936) questioned the existence of a specific enzyme and proposed that the oxidation of ascorbic acid in plants is catalyzed chiefly by copper ions. Stotz, and coworkers (1937) also presented the argument that ascorbic acid is oxidized by ionic copper or by copper in combination with non-specific proteins, and that it is unnecessary to postulate an ascorbic acid oxidase. Their arguments have been disposed of by the demonstration of the far greater catalytic activity of enzyme preparations than of nonspecific copper proteins per unit of copper. Ramasarma, et al. (1940) pointed out that the high activity of their enzyme preparation was inexplicable without the assumption of a specific enzyme as catalyst. Lovett-Janison and Nelson (1940) found their preparation from squash to be 1100 times as active as copper alone, 13,000 times as active as copper-albumin and 4,100 times as active as copper-gelatin, when activities were compared on a copper basis.

Ascorbic acid oxidase has been reported in a variety of plants including cabbage, apple, squash, barley, pumpkin, cucumber, banana, turnip, parsnip, potato, lettuce, melon, cauliflower, peach, watercress, pea, bean, sweet corn, chard, carrot, spinach and the pods of the drumstick tree, Moringa pterygosperma. It is possible that some of these observations involved an indirect oxidation of ascorbic acid. There is little doubt that ascorbic acid oxidase could be demonstrated in many more types of plants. As stated, however, there is no evidence for its occurrence in animals.

Powers, et al. (1944) described the preparation of what they considered to be the most highly purified ascorbic acid oxidase that had been isolated. The juice from the rinds of 20 bushels of yellow summer squash was clarified with 0.3 saturated ammonium sulfate and the enzyme was then precipitated at 0.6 saturation. The precipitate was dissolved in phosphate buffer and fractionated with magnesium sulfate. The enzyme was adsorbed on alumina and eluted with phosphate buffer. Treatments with acetone and lead subacetate were followed by another adsorption on alumina and elution. The purified preparation was blue and precipitated upon dialysis. Its Q_{O_2} was approximately 1,000,000. When the enzyme was diluted it was necessary to add an inert protein like gelatin to prevent rapid inactivation. Diemair and Zerban (1943) have achieved a 130 fold purification of ascorbic acid oxidase from pumpkin. Tadokara and Takasugi (1939) reported the preparation of crystalline ascorbic acid oxidase; however, the activity was far lower than that of other non-crystalline preparations. Considerable doubt remains that the enzyme was actually obtained in a crystalline state (consult critique of Dawson and Mallette, 1945).

Although studies with inhibitors had earlier suggested a role for copper, the first substantial evidence that ascorbic acid oxidase is a copper-protein was presented by Ramasarma, et al. (1940). They observed that the activity and the copper content increased in a nearly parallel manner during purification of the enzyme. Their best preparations had only about 0.03% copper. Stotz (1940) obtained a considerably purer preparation from cucumber; it had 0.25% copper and its action was inhibited by sodium diethyldithiocarbamate

and by cyanide. Lovett-Janison and Nelson (1940) described an even more active preparation which contained 0.15% copper and was isolated from squash. The copper content and enzymatic activity were parallel after the preliminary steps in the purification; this observation was also verified by Meiklejohn and Stewart (1941) and by Diemair and Zerban (1943). Meiklejohn and Stewart reported that dialysis against NaCN inactivated the enzyme, but that the addition of a copper salt restored the activity. The purest preparation of Powers, et al. (1944) contained 0.24% copper.

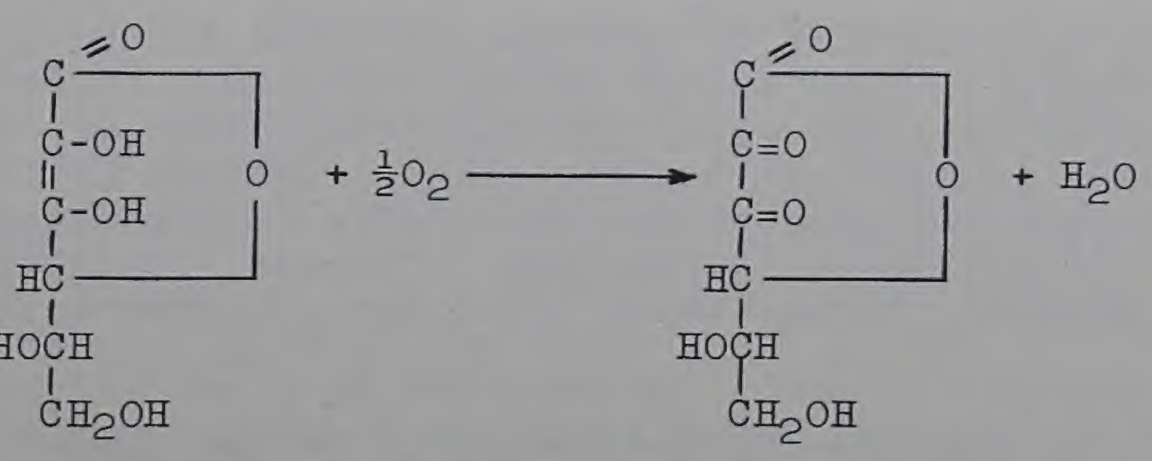
It is reported that in addition to ascorbic acid the oxidase will attack d-glucoascorbic acid (Johnson and Zilva, 1937); however, it has no effect on pyrogallol, p-phenylenediamine, leucoindophenol and p-cresol, and only slight activity on catechol and hydroquinone (Lovett-Janison and Nelson, 1940).

There has been a wide variation in the reported pH optimum of ascorbic acid oxidase; the values have ranged from pH 4.6 to 6.6. Powers, et al. (1944) observed that their highly purified enzyme had a pH optimum of 5.6 in citrate buffer. The enzyme is inactivated by 10 minutes heating at 70° C. (Matukawa, 1940). It is also inactivated during the course of its enzymatic action. This inactivation can be minimized by the addition of an inert protein such as gelatin or by the addition of small amounts of catalase or peroxidase (Powers and Dawson, 1944). These authors suggest that the inactivation arises chiefly from some precursor of hydrogen peroxide which has a transitory existence.

The early reports on ascorbic acid oxidase were in disagreement on the inhibition of the enzyme by cyanide; Szent-Györgyi's (1931) "hexoxidase" was rather insensitive to this inhibitor. In contrast, the purified enzyme preparation of Srinivasan (1936) was inhibited by 10^{-5} M cyanide and strongly inhibited at 10^{-4} M. Diemair and Zerban (1944) also observed strong inhibition by cyanide and sulfide at 10^{-4} M concentrations and by carbon monoxide. Stotz, et al. (1937) noted inhibition of ascorbic acid oxidation by such copper inhibitors as sodium diethyldithiocarbamate, 8-hydroxyquinoline and potassium ethylxanthate.

Szent-Györgyi (1931) conceived of ascorbic acid serving the role of an electron carrier in respiration. He observed that ascorbic acid could be alternately oxidized enzymatically and reduced by glutathione. This concept was expanded by Hopkins and Morgan (1936).

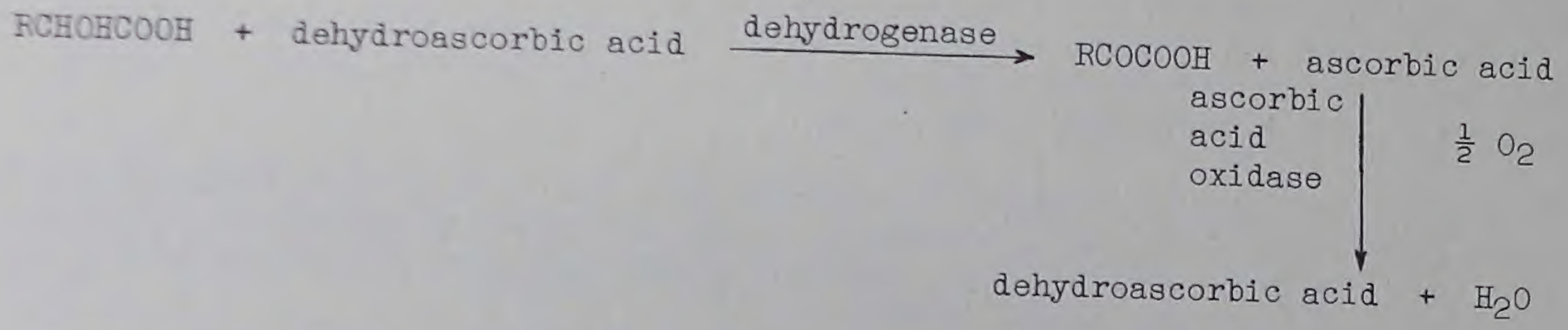
St. Huszák (1937) suggested that hydrogen peroxide is formed by ascorbic acid oxidase, and then peroxidase functions in conjunction with it. Upon the demonstration that ascorbic acid oxidase was a copper-protein, the hydrogen peroxide question was reopened, for the other oxidases of comparable nature had been found not to produce hydrogen peroxide. Steinman and Dawson (1942) could find no evidence of hydrogen peroxide in the ascorbic acid oxidase reaction mixtures. Apparently, however, hydrogen peroxide is formed during the oxidation of ascorbic acid by copper ions. The action of ascorbic acid oxidase involves the formation of dehydroascorbic acid:



ascorbic acid — dehydroascorbic acid (may occur as the hydrate)

Boswell and Whiting (1940) were inclined to discount the importance of ascorbic acid oxidase in the respiration of plants, however, an important role has been ascribed to it by others. James and Cragg (1941, 1943) reported that the addition of lactic, glycolic or tartaric acids increased the oxygen consumption of barley saps in the presence but not in

the absence of ascorbic acid. They believed that the substances served as electron donors to keep ascorbic acid in the reduced state. The ascorbic acid oxidase thus served as a terminal oxidase.



It is difficult to say what percentage of a plant's respiration may follow such a pathway, but it would not be surprising if a cyclic oxidation-reduction by way of ascorbic acid constituted a major part of the respiration of many plants. As with tyrosinase, the ultimate electron donors are not too well defined, but the data of James and Cragg certainly implicate the alpha-hydroxy acids in this capacity.

PEROXIDASES

Peroxidases are enzymes which in the presence of hydrogen peroxide catalyze the oxidation of many phenols and aromatic amines. In addition the peroxidase from several plants has been shown to serve as an oxidase when dihydroxymaleic acid is furnished as a substrate. The early studies of peroxidases were admirably reviewed by Kastle (1910). Much confusion as to the nature and distribution of peroxidases resulted because early workers used a qualitative, non-specific test for them, such as the coloring of guaiacum, benzidine, or p-phenylenediamine in the presence of hydrogen peroxide. Unfortunately a positive reaction is given by any hematin compound (Bancroft and Elliott, 1934). The purification and crystallization of peroxidases in recent years has aided in securing accurate information on their nature and distribution. At one time peroxidases were thought to be almost universally present in tissues of animals; although this is now doubted, Bancroft and Elliott (1934) did find peroxidase in lung and spleen but questioned its importance in any other organs. The enzyme is widely distributed in plants and bacteria.

A crystalline peroxidase (lactoperoxidase) from milk (Theorell and Paul, 1944) and a highly purified peroxidase (verdoperoxidase) from leucocytes (Agner, 1941) are as yet the only animal peroxidases that have been recovered in a pure state. These animal peroxidases, in contrast to the protohematinprotein peroxidases isolated from plants, contain green hematins of unknown constitution. Most of the knowledge concerning peroxidases has been obtained with horse radish peroxidase, and most of the discussion will be limited to this enzyme. Verdoperoxidase, lactoperoxidase and cytochrome c peroxidase will be treated separately.

Substrates which are oxidized by hydrogen peroxide in the presence of peroxidase include o-phenylenediamine, leucomalachite green, benzidine, bilirubin, and many phenolic compounds such as pyrogallol, guaiacol, hydroquinone, catechol, o-, m-, and p-cresol, tyrosine and adrenaline (Sumner and Somers, 1947). Elliott (1932b) concluded that peroxidase oxidized practically all phenols but was unable to demonstrate the oxidation of the following physiologically important compounds: formate, lactate, acetate, oleate, glucose, fructose, glycerol, ethanol, formaldehyde, glycine, glutamic acid, phenylalanine, histidine, or brucine.

Peroxidase activity has usually been measured by the amount of pyrogallol oxidized in the presence of hydrogen peroxide and the enzyme during a five minute period (Willstatter and Stoll, 1918). The enzyme is inactivated with sulfuric acid at the end of the test; the purpurogallin formed is extracted with ether and its quantity determined colorimetrically. Sumner and Gjessing (1943) have modified the original method and recommend a smaller reaction volume and a higher concentration of hydrogen peroxide. The purity of peroxidase is expressed as the purpurogallin number (Purpurogallin Zahl, P. Z.). This is the number of mg. of purpurogallin formed per mg. of dried enzyme preparation when the enzyme acts upon 5 g. of pyrogallol in 2 liters of water plus 10 ml. of 0.5% hydrogen peroxide for 5

minutes at 20° C. Some workers have determined peroxidase activity by manometric measurement of the oxygen liberated from the hydrogen peroxide remaining after the peroxidase reacted for a given period in the presence of the substrate. Elliott (1932a) employed manganese dioxide whereas Randall (1946) and Altschul and Karon (1947) used catalase to decompose the residual peroxide.

Reports from various laboratories of early attempts to purify peroxidase gave very inconsistent descriptions of the properties of the enzyme. Willstatter and Pollinger (1923) reported a peroxidase preparation with a P. Z. of 3070 but found no relation between its activity and its iron content. On the other hand, Kuhn, Hand and Florkin (1931), with preparations of similar and lower P. Z., found a close parallelism between enzymatic activity and total hematin content. In turn, Elliott and Keilin (1934) found no strict proportionality between enzyme activity and total hematin content of various peroxidase preparations. They reported that a preparation of P. Z. 818 contained 1.05% hematin, whereas Kuhn, et al. (1931) found only a tenth this amount of hematin in a preparation of P. Z. 3,400.

It was not until Theorell (1942, 1943) obtained crystalline horse radish peroxidase that the hematin content and its relation to activity could be definitely established. Theorell employed ammonium sulfate and alcohol fractionation in conjunction with electrophoresis to prepare the crystalline enzyme. The activity of Theorell's crystalline peroxidase was always about P. Z. 900. The hematin content of this crystalline peroxidase was 1.48%, a value which agreed well with that calculated from the molecular weight of peroxidase.

In the course of purifying peroxidase Theorell found that the active enzyme could be split into two components. One component, peroxidase 1 or paraperoxidase, moved toward the cathode at pH 7.5. This component was not present in constant amounts in different preparations and appeared to be extremely labile. Its absorption spectrum was typical for a parahematin. Its activity in the purpurogallin test was the same as that of peroxidase (Theorell, 1947). The second component, peroxidase 2, migrated toward the anode. Peroxidase 2 was crystallized and was used in further studies of the properties of peroxidase.

The occurrence of hematin in peroxidase has been recognized for some time, however, conclusive proof that protohematin is the prosthetic group of peroxidase has been presented only recently. Keilin and Mann (1937) gave spectroscopic and other evidence that hematin was associated with horse radish peroxidase, but it was not until Theorell (1940) reversibly split peroxidase that protohematin was conclusively shown to be the prosthetic group. He treated a highly purified peroxidase solution with a cold mixture of acetone and hydrochloric acid. The colorless protein apoenzyme was precipitated and the protohematin went into solution. The protein component was sedimented by centrifugation, dissolved in cold 1% sodium bicarbonate and then treated with a solution of pure blood protohematin dissolved in a minimum amount of alkali. After about 30 minutes the pure brown color of peroxidase appeared and 93% of the original activity was regained. This procedure allowed workers to investigate the specificity of the prosthetic group for peroxidase activity. Gjessing and Sumner (1942) found that almost complete activity was recovered when protohematin or mesohematin was substituted for the protohematin removed from the protein, whereas slightly less activity was regained with hematohematin. They were unable to obtain peroxidase activity with nickel, cobalt, and copper protoporphyrins, but manganese protoporphyrin at one concentration gave 20 to 30% activity. This activity of manganese has been questioned by Theorell (1945). Theorell, Bergstrom, and Akeson (1943) found that protohematin recombined mole for mole with the apoenzyme of peroxidase, and almost full activity was recovered. The enzymes reconstructed with deuterohematin and mesohematin were less active than with protohematin, and hematohematin was found to be inactive by these workers. Any changes in the propionyl groups in positions 6 and 7 of the hematin destroyed its ability to form active peroxidases with the apoenzyme. Theorell (1947) concluded that acid groups were needed in both the 6 and 7 positions and that the length of these carbon side chains was important.

The light absorption curve of crystalline peroxidase is similar to that of methemoglobin and according to Theorell (1942) shows maxima at 260, 402, 500, and 640 mμ with a slight intensification at 380 and 550 mμ. Keilin and Mann (1937) observed that a peroxidase preparation gave four absorption bands at 645, 583, 548 and 498 mμ; the band at 583

was very faint. At pH 11 the four bands were replaced by two bands at 583 and 549 mμ. Upon reacidifying the solution the four banded spectrum reappeared. Reduction of peroxidase with sodium hydrosulfite gave a red solution of ferroperoxidase with bands at 594 mμ and 558 mμ; upon shaking with air the ferroperoxidase was reoxidized. Carbon monoxide formed a compound with the reduced peroxidase resembling that of carboxyhemoglobin.

Potassium cyanide, which inhibits peroxidase completely at a concentration of 0.001 M, altered the spectrum of the enzyme so that it was very similar to that of cyanohemoglobin. Sodium fluoride, nitric oxide, and hydrogen sulfide produced other characteristic changes in the spectrum. Sodium azide and hydroxylamine, which inhibit peroxidase action by about 40% at 0.001 M, produced no change in the spectrum of peroxidase in the absence of hydrogen peroxide. In the presence of hydrogen peroxide, however, sodium azide caused a change in the spectrum of peroxidase; the iron remained in the ferric state.

Peroxidase apparently forms three compounds with hydrogen peroxide. When equivalent amounts of hydrogen peroxide and peroxidase are mixed the compound designated by Theorell (1942) as "H_2O_2-peroxidase I" is formed. This compound is greenish in color and is characterized by a sharp band at 658 mμ. Very soon however the color begins to change to red as the bands which Keilin and Mann (1937) first observed begin to appear at 561 and 530 mμ. This compound is designated as "H_2O_2-peroxidase II" by Theorell (1947). The green and the red compound each gives instantly the normal peroxidase spectrum on addition of hydroquinone. A third compound is formed with peroxidase in the presence of ten to twenty moles excess of hydrogen peroxide; this compound has strong absorption bands at 583 and 545 mμ and has been numbered "III" by Theorell.

Horse radish peroxidase consists of one molecule of ferriprotoporphyrin linked to a colorless protein. The molecular weight is 44,100 (Theorell, 1942a). Theorell and Åkeson (1943) have analyzed the nitrogenous fractions of horse radish peroxidase and have reported the following values: humin nitrogen 1.1%, amide nitrogen 13.0%, cathode nitrogen 24.2%, neutral nitrogen 45.1%, anode nitrogen 16.2%, histidine 0.71%, arginine 6.91%, and lysine 4.06%. The information available at the present concerning the nature of the hematin linked groups in horse radish peroxidase has been summarized by Theorell (1947).

Chance (1943) studied the kinetics of peroxidase action by use of the Hartridge-Roughton flow method and reported that peroxidase very rapidly forms a complex with hydrogen peroxide. The velocity constant for the formation of this complex was 1.2×10^7 liter $mole^{-1}$ $sec.^{-1}$; this value is very similar to that for the combination of muscle hemoglobin and oxygen. Although no spectroscopic evidence could be found, kinetic data indicated a bimolecular combination of the enzyme-H_2O_2 compound and the acceptor. Chance concluded that in the range of experimental concentrations, peroxidase action involved a second order combination of enzyme and substrate followed by a first order decomposition. Theorell (1947) believed that his view, that the green "peroxidase-H_2O_2 I" was the primary reaction product and was the physiologically active one, was not invalidated by Chance's data.

Peroxidase iron is considered to remain in the ferric state during catalytic activity, since peroxidase is not inhibited by carbon monoxide as a ferrous compound would be. However, Swedin and Theorell (1940) presented evidence that peroxidase functions as an aerobic oxidase with dihydroxymaleic acid as substrate. No other substrate has been found to act in this capacity. The light reversible inhibition of the reaction by carbon monoxide furnished evidence of a valence shift of the iron to the ferrous state during the oxidation.

Agner (1941, 1943) isolated from leucocytes an enzyme with peroxidatic activity. This enzyme has been named verdoperoxidase. Agner has prepared verdoperoxidase with a P. Z. of 75 from empyemic fluid, which serves as a convenient source of leucocytes. This preparation was completely homogeneous upon cataphoresis, so apparently verdoperoxidase is less than one tenth as active as pure horse radish peroxidase.

Verdoperoxidase when oxidized has absorption bands at 690, 625, 570 and 500 mμ and may be reversibly reduced to give bands at 637 and 475 mμ. Hydrogen peroxide, cyanide, and hydroxylamine form compounds with oxidized verdoperoxidase, whereas azide forms a compound with the reduced enzyme. Carbon monoxide and fluoride do not give spectroscopically observable compounds. The absorption spectrum of verdoperoxidase and data from titration

of the iron of the enzyme with hydrosulfite indicate that the prosthetic group is probably composed of an iron porphyrin. Unlike catalase and peroxidase, verdoperoxidase is not split in an acid acetone mixture, so that a final identification of the prosthetic group has not been made.

Verdoperoxidase readily oxidizes hydroquinone, p-phenylenediamine, catechol, pyrogallol, and ascorbic acid in the presence of hydrogen peroxide. Resorcinol and benzidine are less easily oxidized and phenol, tyrosine, and ethyl alcohol are not oxidized. Its action is inhibited by cyanide, hydroxylamine, azide and fluoride, but not by carbon monoxide. The enzyme is remarkably resistant to alkali, acid, alcohol, and formalin.

Peroxidase has long been known to occur in milk, but it is only recently that it has been established as distinct from leucocyte and other peroxidases. Elliott (1932b) prepared crude milk peroxidase and showed that it oxidized nitrite and tryptophane, whereas horse radish peroxidase did not. Theorell and Akeson (1943) obtained from cow's milk a highly purified peroxidase (lactoperoxidase) with P. Z. 71.5. Solutions of this enzyme were greenish and had absorption bands at 640, 600, 550, 500, 413, and 280 mμ. Theorell and Paul (1944) later reported the crystallization of this enzyme.

Lactoperoxidase is reduced by sodium hydrosulfite and gives characteristic compounds with fluoride and cyanide. Compounds corresponding to those observed by Keilin and Mann (1937) (Theorell's H_2O_2-peroxidase II) with horse radish peroxidase are formed when lactoperoxidase is treated with the proper amounts of hydrogen peroxide. Theorell and Akeson (1943) observed that the enzyme gave a hemochromogen reaction different from that of protohematin. Theorell and Pederson (1944) found the molecular weight of lactoperoxidase to be 93,000 and the iron content to be 0.070%. Thus one atom of iron occurs per molecule of enzyme.

A peroxidase isolated from yeast, which acts only on reduced cytochrome c, has been investigated by Abrams, et al. (1942). In the presence of hydrogen peroxide it oxidizes ferrocytochrome c to ferricytochrome c. The enzyme is apparently a hematin protein as the activity is proportional to the hematin content and to light absorption at the wave length of the Soret band. Cytochrome c peroxidase has absorption bands at 410, 500, and 620 mμ and forms a stable complex with hydrogen peroxide with bands at 420, 530, and 560 mμ.

CATALASE

Catalase is an enzyme which catalyzes the decomposition of hydrogen peroxide to water and molecular oxygen:

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

Catalase is a metalloprotein enzyme with hematin as its prosthetic group. Hydrogen peroxide is decomposed at increasingly faster rates by simple iron salts, hematin, and by catalase; the reaction with catalase is an extremely vigorous one. Stern (1941) has discussed the relative catalytic activity of these and many other hematin containing compounds.

The specificity of catalase for the decomposition of hydrogen peroxide has been frequently emphasized. However, Keilin and Hartree (1945b) have presented evidence that catalase may in addition function in coupled oxidations. They observed the oxidation of alcohols to aldehydes in the presence of catalase and hydrogen peroxide slowly generated by chemical reactions or furnished by the action of an enzyme such as xanthine oxidase.

$$RCH_2OH + H_2O_2 \longrightarrow RCHO + 2\ H_2O$$

Methanol, ethanol, n-propanol, isobutanol, and ethylene glycol were shown to undergo oxidation. Keilin and Hartree (1945b) were led to conclude that "it is therefore unlikely that the main function of catalase consists in the decomposition of H_2O_2 into O_2 and water in order to protect cells from its toxic effect. The catalysis of coupled oxidations by means of H_2O_2 formed in a primary oxidation is a more likely biological property of catalase."

Theorell (1948) also reports the oxidation of alcohols to aldehydes by hydrogen peroxide plus catalase but prefers to term the reaction a peroxidatic one rather than a coupled oxidation. The demonstration of the formation of an intermediate compound of catalase and hydrogen peroxide and its rapid reaction with lower alcohols supports this concept that catalase acts as a peroxidase. Theorell also is inclined to believe that the chief physiological role of catalase is peroxidatic and that the destruction of peroxide to form water and oxygen is of secondary importance.

The fact that plant and animal tissues decomposed hydrogen peroxide was observed early in the nineteenth century, but it was not until the turn of the present century that Loew (1901) attached the name catalase to the specific enzyme involved. Catalase has an almost universal distribution in living organisms and Loew made the statement, "There does not exist a group of organisms or any organ or even a single vegetable or animal cell that does not contain some catalase, as far as the observations of the writer go." Further work has revealed that catalase is absent only from the obligate anaerobic bacteria and in certain microaerophilic bacteria.

Catalase activity has generally been measured by determining the amount of hydrogen peroxide remaining after catalase has acted for a definite period of time. Measurement of the volume of oxygen produced in the reaction may also be used. Many workers have employed the procedure of von Euler and Josephson (1927) or some modification of it. In this procedure the properly diluted catalase is allowed to react with approximately 0.01 N H_2O_2 at pH 6.8 and at 0° C. Aliquots of the reaction mixture are removed at 0, 3, 6, 9, and 12 minutes time, inactivated with 2N H_2SO_4 and the residual H_2O_2 titrated with permanganate. The monomolecular k values are calculated ($k = \frac{1}{\text{time in min.}} \times \log_{10} \frac{\text{initial amt. substrate}}{\text{final amt. substrate}}$; the conditions employed are those listed above), and the k value for zero time is found by extrapolation. Sumner and Somers (1947) recommended that the enzyme concentration should be adjusted so that the initial value of k is greater than 0.025 but less than 0.04. The purity of catalase preparations is usually expressed as Kat. f. (Katalase fahigkeit or catalase capability), which is obtained by the equation:

$$\text{Kat. f.} = \frac{k\ (\text{initial})}{\text{grams of enzyme employed in test}}$$

Catalase activity determinations are complicated by the inactivation of catalase during the decomposition of hydrogen peroxide. Bonnichsen, et al. (1947) found that when they increased the concentration of catalase 50 fold and employed a method of rapidly following the decomposition of hydrogen peroxide, inactivation was greatly decreased. In their determinations the activity values were independent of variations of initial substrate concentration, were only slightly influenced by temperature changes, and gave a constant activity for a wide range of enzyme concentrations.

Catalase was crystallized for the first time by Sumner and Dounce (1937a) from beef liver; it was the first enzyme containing iron to be obtained in crystalline form. Later many workers isolated crystalline catalase from different kinds of liver (Dounce, 1942; Agner, 1943a; Bonnichsen, 1947a), from beef erythrocytes (Laskowski and Sumner, 1941; Agner, 1943b), and from bacteria (Herbert and Pinsent, 1947).

Sumner and Dounce (1937b) prepared crystalline catalase by extracting beef liver with 35% dioxane. Additional portions of dioxane were added and the material that precipitated in the cold was discarded. After addition of saturated ammonium sulfate until the dioxane solution became hazy, the catalase quickly crystallized. Recrystallization from phosphate buffer solution (initial pH 7.4) was achieved by shifting the pH to 5.3. The first crystallization yielded fine needles, but recrystallization gave thin plates or prisms. Crystals were later obtained from acetone and alcohol extracts (Dounce, 1942), and from dioxane without the addition of ammonium sulfate (Sumner and Dounce, 1939).

Agner's (1943a) method for preparing crystalline catalase differed substantially from that of Sumner and Dounce. He ground horse liver, extracted it with water and then added ethyl alcohol and chloroform, removed the precipitate, and concentrated the solution. The addition of acetone to 40 volume per cent brought down most of the catalase. The precipi-

tate was dissolved in water and subjected to fractional precipitation with ammonium sulfate and then with ethyl alcohol. These two precipitations were repeated. The dialyzed preparation was adjusted to pH 4.7 and the precipitate was removed. Crystals were obtained when the solution was brought to 46% saturation with ammonium sulfate.

From the values reported in the literature the activity of crystalline and apparently pure catalases would seem to vary with the source of enzyme and the method of preparation. Sumner and Dounce (1937a) originally reported a Kat. f. of 43,000 but later (1937b) revised this value downward to 25,000 to 26,000. In further studies Sumner, Dounce and Frampton (1940) found the Kat. f. of crystalline beef liver catalase to vary from 28,000 to 35,000 while that of crystalline horse liver catalase varied from 22,000 to 55,000. Agner (1943a) regularly prepared horse liver catalase with a Kat. f. of about 61,000. He believed the variation in activity for catalase from the same animal species, reported by different authors, lay in the method of preparation of the enzyme. Bonnichsen, et al. (1947a) using a method designed to eliminate inactivation of the enzyme during measurement, reported activities for horse blood and liver catalases corresponding to a Kat. f. of 61,000 and 52,000 respectively. Theorell (1948), employing this same method, reported that the Kat. f. of all catalases investigated was about 80,000 when they had been freed of biliverdin.

Zeile and Hellstrom (1930) indicated that the active group of catalase was a hematin. Later Keilin and Hartree (1936) confirmed this observation, and Stern (1936) established the identity of the prosthetic group with the protohematin of hemoglobin. Stern isolated the crystalline methyl ester of the porphyrin derived from catalase and in addition showed that the hematin from catalase could be recombined with horse globin to form hemoglobin. Catalase, hemoglobin, cytochrome c and peroxidase each possesses hematin as its prosthetic group.

Certain catalase preparations have been shown to have, besides hematin, a blue component which may be split off by treatment with acetone and hydrochloric acid. Lemberg and Wyndham (1937) isolated crystalline biliverdin hydrochloride from crystalline liver catalase. They believed that the biliverdin was not present in the catalase preparations as such but as a bile pigment hematin compound bound to a protein. In view of the constant molar ratio in horse liver catalase of biliverdin to hematin (about 1:3) Lemberg, et al. (1939) assumed that the bile pigment hematin formed an essential part of the molecule. However the following considerations lead to the conclusion that the biliverdin content is unimportant for catalase activity. (1) Sumner, et al. (1940) and Lemberg and Legge (1943) observed that the specific activity of catalases increased as the ratio of blue component to hematin decreased. (2) Catalases from beef, horse, and human blood, human liver, and horse kidney are free of biliverdin. Indeed, Theorell (1948) suggested that the biliverdin content of liver catalases is a postmortem artifact, since Bonnichsen was able by using suitable preparative procedures to prepare liver catalases free from biliverdin.

Agner (1943b) observed that crystalline erythrocyte catalase had absorption bands at 626, 525, and 500 mu. These wavelengths agreed well with those found by the same author for liver catalase. He reported, however, that the absorption curve varied with the type of buffer used. This may explain the variations recorded in the literature for the absorption maxima of catalase preparations. Stern (1937) made a rather complete spectroscopic study of catalase using less pure preparations. Bonnichsen (1947b) found the absorption constant, β, for crystalline horse kidney catalase to be 84.5×10^7 cm^2 mM^{-1} and for crystalline human liver catalase to be 67×10^7 cm^2 mM^{-1} at 405 mμ. In contrast to other hemoproteins, such as methemoglobin, catalase is not reduced by sodium hydrosulfite or other common reducing agents. Zeile, et al. (1940) have reported being able to reduce catalase reversibly by saturating the solution with hydrogen sulfide and then adding sodium hydrosulfite. The hydrogen sulfide was removed with an indifferent gas leaving reduced catalase. The catalase was oxidized by shaking the reduced solution in air.

A variety of derivatives of catalase hematin have been described. Keilin and Hartree (1936) found that catalase formed spectroscopically observable compounds with sodium azide, hydroxylamine, sodium fluoride, nitric oxide, ammonia, hydrazine hydrate, potassium cyanide, hydrogen sulfide and ethyl hydroperoxide. They classified the inhibitors as those (potassium cyanide, hydrogen sulfide, etc.) which prevented the formation of their postulated

reduced catalase-peroxide compound and those (azide, hydroxylamine, hydrazine) which stabilized this reduced intermediate. Recently Agner and Theorell (1946) showed that phosphate, chloride, sulfate, fluoride, formate, acetate, lactate, propionate and hippurate were able to combine with catalase iron; in sufficiently high concentrations at low pH's they inhibited catalase activity.

Observations at various pH's of the changes in absorption of light by catalase solutions in the presence of phosphate, acetate, and formate led Agner and Theorell (1946) to conclude that an hydroxyl ion was bound to the catalase hematin iron. Thus the inhibiting anion had to compete with the hydroxyl for the bond to the iron; this explained why anion inhibition was increased with decreasing hydroxyl concentration. The dissociation constant for catalase iron and hydroxyl was calculated as $1 \times 10^{-10.2}$. The dissociation constant for acetate and catalase iron was $1 \times 10^{-2.5}$ and that for formate and catalase iron was $1 \times 10^{-5.4}$.

Sumner and Dounce (1937a) reported that their crystalline catalase had about 0.10% iron. Agner (1943a) found the iron content of horse liver catalase (Kat. f. 60,000) to be 0.093; this corresponds rather closely to the value of 0.09% listed by Sumner, and coworkers (1940) for beef and horse liver catalases of lower Kat. f. Bonnichsen (1947b) reported similar values for horse kidney and horse and human blood catalases. Agner's (1938, 1943a) analyses revealed that his horse liver catalase preparations contained 0.021% copper, but Sumner and Dounce (1939) found only 0.0015% copper in their catalase. Agner (1938) was able to separate catalase preparations by precipitation with picric acid into one fraction which contained 0.1% iron and little copper and one which contained 0.16% copper and very little iron. The activity of either fraction was considerably lower than that of the whole. It still remains questionable that copper has any function in the action of catalase; certainly no specific role has yet been established.

The specificity of the reaction mediated by catalase depends upon the protein moiety and upon how this is attached to the hematin groups. Theorell and Akeson (1943) analyzed horse liver catalase for its humin, amide, anodic, neutral and cathodic nitrogen content, as well as for its percentage histidine, arginine, and lysine. Bonnichsen (1947b) believed that since the hematin content of horse blood catalase differed from that of horse liver catalase (he reported that horse blood catalase has four hemin groups and horse liver catalase has three hemin groups and one verdohemochromogen group per molecule) there might also be a difference in the protein of the two enzymes. However the nitrogen distribution and histidine, arginine, lysine, tyrosine, cystine, glutamic and aspartic acid content were found to agree within limits of error between the two enzymes. He was also unable to demonstrate any serological differences between the two enzymes. These data would suggest the identity of the protein of horse liver catalase and horse blood catalase.

Sumner and Gralen (1938) found the molecular weight of beef liver catalase to be 248,000. Later Sumner and coworkers (1940) altered this value to 225,000. Agner (1938) reported 225,000 for the molecular weight of horse liver catalase. If the catalase of horse liver is assumed to contain four iron atoms per molecule and to have 0.093% iron, its calculated molecular weight is 240,000. A molecular weight of 225,000 would correspond to an iron content of 0.099%.

Sumner and Dounce (1937b) reported the isoelectric point of beef liver catalase to be 5.7, and Stern (1932) reported 5.58. More recently Agner (1943a) found the isoelectric point of horse liver catalase to be 5.4.

A study of the mechanism of the catalytic reaction between catalase and hydrogen peroxide has been hindered by the very speed with which the reaction takes place. As expressed by Keilin and Hartree (1945a), the reaction consists in a violent, almost explosive decomposition of hydrogen peroxide during which it is almost impossible to observe any changes in color and absorption spectrum of the enzyme. Although the enzyme is rather rugged, it is partially inactivated during its period of catalytic activity; the reaction rate either decreases throughout the reaction or stabilizes at a rate much less than its initial value.

Keilin and Hartree (1938, 1945a) suggested that the decomposition of hydrogen peroxide by catalase involves a change in the valence of the catalase iron and an oxidation of the ferrous catalase by molecular oxygen according to the equation:

$$4\ Fe^{+++} + 2\ H_2O_2 \longrightarrow 4\ Fe^{++} + 4\ H^+ + 2\ O_2$$
$$4\ Fe^{++} + 4\ H^+ + O_2 \longrightarrow 4\ Fe^{+++} + 2\ H_2O$$
$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

This reaction mechanism was developed from studies of azide-catalase acting on hydrogen peroxide. Such a system was advantageous, for in the presence of azide, catalase has a stabilized ferrous form which was accepted as a useful model of free catalase. Keilin and Hartree (1938, 1945a) cited impressive evidence that the ferric iron of azide-catalase was reduced by hydrogen peroxide to an autooxidizable ferrous compound. Their method (Keilin and Hartree, 1938) of demonstrating the necessity of oxygen for the reaction was later (Keilin and Hartree, 1945a) shown to be in error, but the function of O_2 was still postulated though not experimentally verified. By analogy with the azide-catalase they reasoned that the reaction of free catalase also involved an oxidation-reduction of the catalase iron. However, whether the mechanism of the reaction of hydrogen peroxide with azide-catalase and free catalase is the same has been questioned. Theorell (1947) believed the mechanisms were different, as azide catalase was inhibited by carbon monoxide whereas free catalase was not.

Sumner (1941) was of the opinion that no valence change of the iron was involved in the action of catalase. He postulated that the following reaction occurred:

$$FeOH + H_2O_2 \longrightarrow FeOOH + H_2O$$
$$FeOOH + H_2O_2 \longrightarrow FeOH + H_2O + O_2$$
$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

Chance (1947) developed a technique for rapidly mixing the components of the catalase system and then recording the absorption spectrum of the mixture oscillographically at extremely short time intervals. With this apparatus he was able to demonstrate an intermediate compound in the catalase-hydrogen peroxide reaction. The enzyme had a high affinity for hydrogen peroxide, and the intermediate formed existed at a constant concentration during catalytic activity. It decomposed slowly in the absence of alcohols and rapidly in their presence. The compound appeared to be a ferric iron-hydrogen peroxide complex. Saturation effects with excess ethanol and methanol led to the conclusion that, as with peroxidase, a further intermediate compound was formed, the velocity constant for the breakdown of which was similar to that for peroxidase. Bonnichsen, et al. (1947) concluded that, since the reaction of catalase and hydrogen peroxide followed a bimolecular course over such wide ranges of enzyme and substrate concentrations, the suggestions by others of complex mechanisms, chain hypotheses, Michaelis hypotheses, and ferri-ferro cycle hypotheses were not supported by their data.

Thus the questions of the mode of action of catalase and whether a valence shift is involved in hydrogen peroxide decomposition remain controversial, and no categorical statement on these matters can be made now.

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Chapter IX

GLYCOLYSIS

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GLYCOLYSIS

Glycolysis is the term used to describe the physiological breakdown of carbohydrates to lactic acid. Since the fermentation of glucose to alcohol and carbon dioxide by yeast follows a similar metabolic pathway, this process will also be discussed in the present chapter. The essential difference between the two processes lies in the means by which the common intermediate, pyruvate, is converted to the end product. In tissues which glycolyze, pyruvate is directly reduced to lactate while in fermentative metabolism pyruvate is decarboxylated and the acetaldehyde thus formed is reduced to ethyl alcohol.

Glycolysis and fermentation are essentially, but not obligatorily, anaerobic processes. By this is meant that the oxidative steps within the process are balanced by the reductive and that no "external" electron acceptor such as oxygen is required. On the other hand, the presence of oxygen does not necessarily abolish either glycolysis or fermentation. Whether oxygen decreases the rate of lactic acid or alcohol production in living cells depends on a number of factors among which are:

1. The amount and activity of terminal respiratory enzymes (Warburg-Keilin system; Cytochrome-cytochrome oxidase system).
2. The relative efficiencies of the aerobic and anaerobic processes in competing for phosphate (Lennerstrand (1936-1937) - Johnson (1941) - Lynen (1941) phenomena).
3. The presence of "uncoupling" agents which release oxidative reactions from accompanying fixation of inorganic phosphate (See Lipmann (1942) and Lardy and Elvehjem (1945) for discussions of the action of dinitrophenol, gramicidin, etc.). A naturally occurring metabolic regulator which functions in this manner has been found in mammalian spermatozoa (Lardy, Ghosh and Plaut, 1949).
4. The influence of oxygen <u>per se</u> on the activity of certain enzymes (Engelhardt-Sakov (1943) phenomenon).

The combined influence of these factors establishes the degree of "oxygen inhibition of fermentative processes" which is Burk's (1939) concise definition of the Pasteur effect.

EARLY DEVELOPMENTS

The over-all equation for the fermentation of hexoses was stated by Gay-Lussac (1810) to be:

$$(1)\quad C_6H_{12}O_6 \longrightarrow 2CH_3CH_2OH + 2CO_2$$

While this equation is still descriptive of the over-all process and may serve as an unattainable goal at which the commercial distiller may aim, it does not take into account the important role of phosphate in making the process a continuous one and in tapping off the free energy of the reaction in a form utilizable for the life processes of the fermenting organism.

The fact that phosphate does play an important role in fermentation was established by Harden and Young (1905-1906) but had been demonstrated, if in a less convincing manner, by earlier investigators including Pasteur and Eduard Buchner*.

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*In this brief discussion sufficient space cannot be devoted to the historical development of our knowledge of fermentation and glycolysis. Students of the subject are urged to become acquainted with the contributions of such men as Lavoisier, Gay-Lussac, Liebig, Bernard, Figuier, Hansen, Pasteur and Traube. For this a good start would be to read the excellent introductory chapter in Harden's (1914) "Alcoholic Fermentation" and the wonderfully enlightening "Der Blutzucker" by Ivar Bang (1913).

Harden and Young found that dialyzing the Buchner cell-free extract of yeast* destroyed its ability to ferment. The addition of boiled yeast juice permitted fermentation to be resumed but only until the phosphate of the yeast juice disappeared. Further additions of phosphate resulted in more fermentation and again the ortho-phosphate disappeared and was fixed in organic form. They were later able to identify the organic ester of phosphoric acid which was formed as Hexose-di-phosphate (hereafter HDP) and formulated fermentation by the cell-free extract as:

$$(2)\quad 2C_6H_{12}O_6 + 2RH_2PO_4 = 2CO_2 + 2CH_3CH_2OH + 2H_2O + C_6H_{10}O_4(PO_4R_2)_2$$

The accumulation of HDP during inanimate fermentation occurs because of the disruption of the highly organized energy utilizing systems which in the living cell assimilate carbohydrate, synthesize protoplasm and carry out other processes necessary for the maintenance of cell life.

From a consideration of the evidence to be presented below, we may conclude that the over-all equation for fermentation either in the cell or extract is:

$$(3)\quad C_6H_{12}O_6 + 2ADP \longrightarrow 2CO_2 + 2CH_3CH_2OH + 2ATP$$

In the Buchner extract the only system capable of utilizing the energy stored in ATP** is that which phosphorylates hexoses. The result is expressed by equation (2).

In the living cell, transfer of high energy phosphate to hexose occurs at a rate sufficient only to keep the fermentation going; the balance of the 2 moles of high-energy phosphate per mole hexose fermented is utilized for life maintenance as described above. Meyerhof (1945) has demonstrated that the fermentation of yeast juice can be changed from the Harden-Young type (equation 2) to that of Gay-Lussac (equation 1) by the addition of a small quantity of adenosinetriphosphatase.

The above considerations bring us to an interesting philosophical point (Johnson, 1942). Since the reactions described by equation (3) are the only source of energy for the yeast cell grown under anaerobic conditions, it follows that all of the energy requirements for growth and reproduction of yeast can be furnished either directly or indirectly by adenosinetriphosphate. In view of the fact that the bulk of the energy liberated during the combustion of carbohydrates by animal tissues is likewise trapped as ATP, (Belitzer and Tsibakova, 1939; Ochoa, 1943; Green, Chapter X) it seems not unlikely that the same might be said for animal tissues.

By analogy with equation (3), the process of glycolysis may be written:

$$(4)\quad C_6H_{12}O_6 + 2ADP \longrightarrow 2CH_3CHOHCOOH + 2ATP$$

That lactic acid can be produced by animal tissues was observed by many early investigators including Claude Bernard. Our understanding of the metabolic factors which influence the ebb and flood of lactic acid in mammalian tissues under various physiological conditions is based on the classic study of Fletcher and Hopkins (1907). While it had previously been known that lactic acid production occurred as a result of muscular activity, these workers were the first to observe that, in an oxygen-free atmosphere, lactic acid accumulated even during rest. They also clearly established the fact that the presence of oxygen resulted in disappearance of lactate from the muscle. Parnas (1915) later reported this disappearance to be a complete oxidation of the lactate. In 1920 Meyerhof discovered the principle of oxidative resynthesis of carbohydrate from lactate by isolated muscle. His data indicated that for every mole of lactate burned to carbon dioxide and

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*For the interesting story of the discovery and the historical and philosophical significance of fermentation by cell-free extracts of yeasts see the Buchners' "Die Zymasegärung" (1903) and the introductory chapter by Harden (1914).

** Abbreviations used here conform with the definitions in Chapter V.

water an additional four to five moles of lactate were resynthesized to glycogen. Thus a cyclic conversion of polysaccharide to lactate during work and of lactate back to polysaccharide during rest was indicated. These observations provided the impetus for a brilliant series of investigations during the following twenty years.

Enzymatic steps in the conversion of glycogen to lactate were elucidated one by one in the laboratories of Embden, Meyerhof, Parnas, Warburg, Cori and Hopkins. These workers identified and occasionally synthesized intermediates which had been found to be formed enzymatically. In this latter aspect of the problem they were joined by Robison, H. O. L. Fischer and Baer, Neuberg and Karrer. The result is that we now can trace the carbon chain of glucose through a series of reactions, each of which is catalyzed by a specific enzyme. These reactions result in the formation of lactate and the conservation of a part of the free energy liberated in the form of pyrophosphate linkages in the universal medium of energy exchange -- Adenosinetriphosphate. The aerobic processes which lead to the formation of high energy phosphate bonds during lactate and pyruvate oxidation are considered in the following chapter.

The glycolytic and fermentative systems, as we know them today are shown schematically in Figure 1.

It seems likely that every molecule of sugar metabolized by animal tissues proceeds through at least a part of the pathway described. Details concerning the various individual reactions and the enzymes which catalyze them will follow.

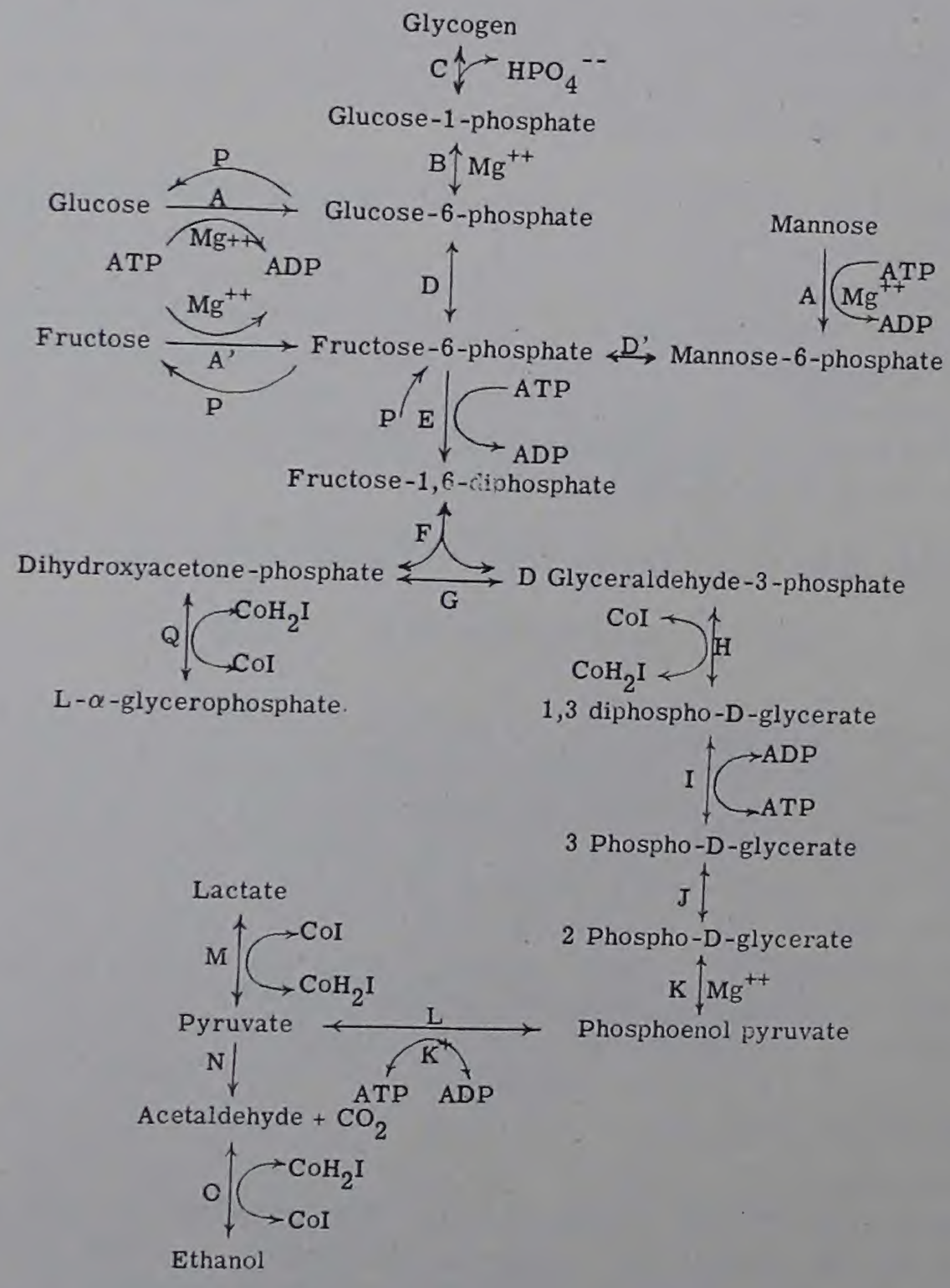
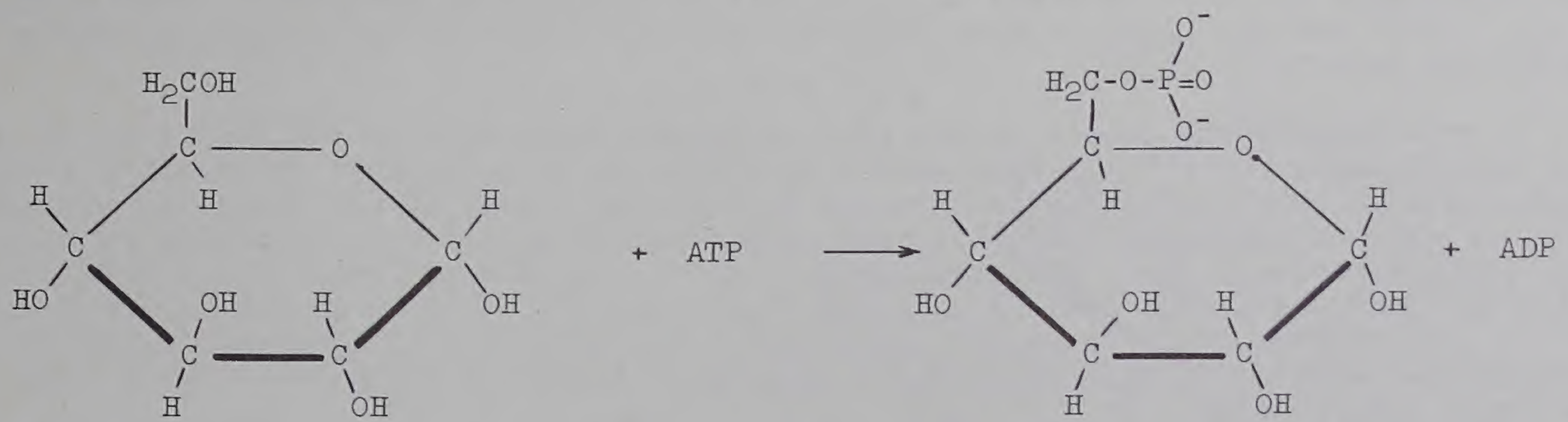


Figure 1

SCHEME OF ANAEROBIC CARBOHYDRATE METABOLISM

A. HEXOKINASE



Glucose

Glucose-6-phosphate
"Robison Ester"*

The enzyme hexokinase carries out a "priming" reaction in that it expends a certain amount of energy in order to prepare the hexoses for attack by other enzymes which will liberate a greater amount of free energy during the breakdown of the molecule. It now appears that with the possible exception of direct oxidation of a minor quantity of glucose to gluconic acid by Harrison's glucose dehydrogenase (see Chapter VI), all sugar metabolized in the animal body must first be phosphorylated. The phosphorylation of glucose at carbon atom No. 6 must precede its glycolysis (via fructose-6-phosphate) or its temporary storage as glycogen (via glucose-1-phosphate). In addition it is possible that the hexokinases may participate in the active absorption of hexoses from the digestive tract by the intestinal mucosa and from the glomerular filtrate by the kidney tubules.

The name hexokinase was given by Meyerhof (1927) to a preparation from autolyzed yeast which initiated glycolysis of glucose in aged muscle extracts. In 1935 Euler and Adler, and, independently, Lutwak-Mann and Mann found an enzyme in yeast which carried out reaction A if Mg ions were also present. Meyerhof (1935) then indicated that the enzyme described by these two groups of workers was responsible for the activity of his original preparation.

Yeast hexokinase has been isolated in crystalline form (Berger, Slein, Colowick and Cori, 1946; Kunitz and McDonald, 1946) as an electrophoretically homogeneous (at pH 5.6 and 6.8) protein having a molecular weight of about 96,000. It acts on glucose and fructose at equal rates but considerably slower on mannose. Mg^{++} is of course necessary for the activity of the enzyme. The crystalline enzyme loses activity when diluted. Concentrated solutions, the presence of glucose or mannose and the presence of other proteins tend to stabilize the enzyme. Insulin seemed the most effective among various proteins tested in protecting the pure hexokinase from denaturation.

The hexokinase of animal tissues is much more labile and therefore difficult to purify but active concentrates have been obtained from brain by Colowick, Slein and Cori (1947). In a preliminary report, G. Cori and Slein (1947) describe the separation of

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*Robison himself (see Robison and MacFarlane, 1941) considered the "Robison Ester" to be the mixture of 70% aldose (glucose and mannose) monophosphates and 30% ketose (fructose) monophosphate isolated from a yeast maceration juice fermentation mixture. Since the term "Embden Ester" has more frequently been used to designate the mixture of hexose monophosphates it seems desirable that it be retained and that we should use Robison's name to designate the glucose-6 ester which he isolated in pure form for the first time.

glucokinase and fructokinase activity from muscle and liver into two distinct fractions. This indicates that mammalian hexokinases are much more specific than the yeast enzyme. A galactokinase has been obtained in an extract of a galactose fermenting yeast by Caputto et al. (1948) and has likewise been demonstrated in extracts of rat intestinal mucosa (Lardy and Feldott, 1948).

A most interesting aspect of the work on animal hexokinase is the report by Price, Cori and Colowick (1945) that this enzyme is inhibited by an extract of anterior pituitary gland and that this inhibition is reversed by insulin. Later it was found that adrenal cortical extract augmented the inhibition of hexokinase by anterior pituitary extracts and that insulin reversed their combined inhibitions (Price, Slein, Colowick and Cori, 1946; Colowick, Cori and Slein, 1947). Detailed investigations of these phenomena are under way in the laboratories of Cori, Stadie and Mirski. The mechanism of hormonal regulation of carbohydrate metabolism will undoubtedly be greatly clarified by future reports from their and other laboratories.

It has been known since the work of Mendel (1929) that DL-glyceraldehyde inhibits glycolysis in tumor, brain and many other tissues utilizing glucose. The inhibitory effect is a property of the L isomer (Needham and Lehmann, 1937). Muscle and liver glycolysis, with either starch or glycogen as the substrate are not specifically inhibited by glyceraldehyde nor is the conversion of HMP or HDP to lactic acid inhibited in a variety of tissue extracts. These observations led Stickland (1941) to investigate the site of glyceraldehyde inhibition more closely. He found DL-glyceraldehyde to inhibit the breakdown of glucose by muscle extracts with yeast hexokinase present to phosphorylate the free sugar. However the addition of a slight excess of hexokinase (above that required to obtain moderately rapid utilization of glucose) was found to prevent the inhibitory action of glyceraldehyde.

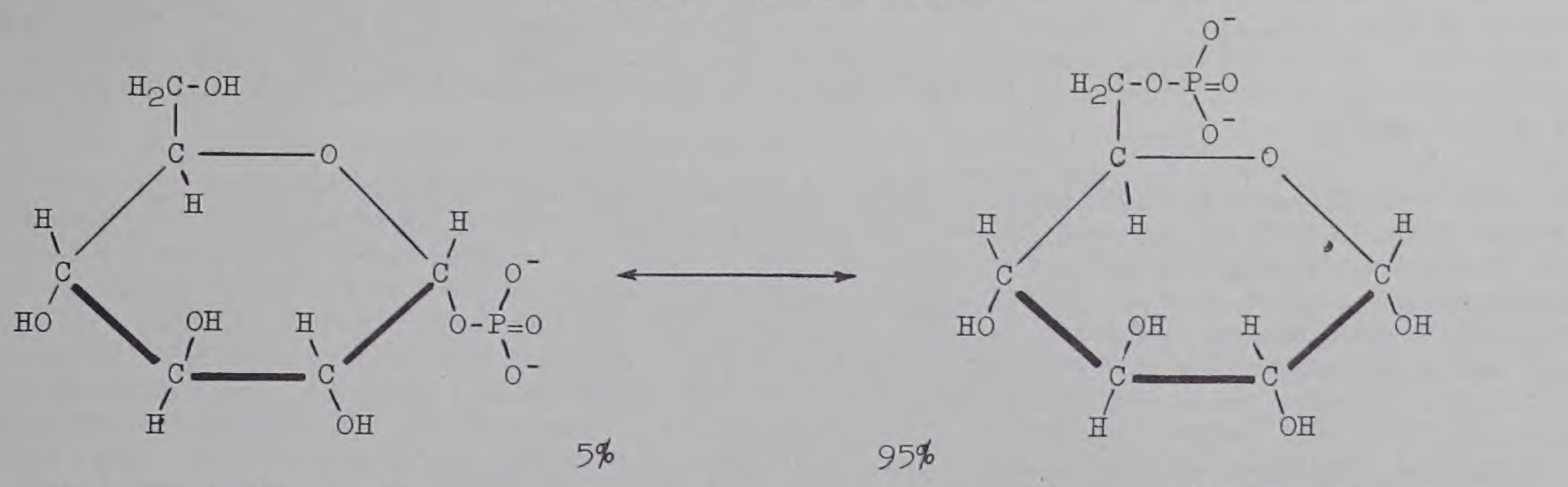
A detailed investigation of the effect of glyceraldehyde on hexokinase has been made in this laboratory (Lardy, Wiebelhaus and Mann, 1947-48). It has been found that the addition of glyceraldehyde to crude tissue extracts does inhibit hexokinase activity. L-glyceraldehyde inhibits hexokinase by virtue of the fact that it combines with dihydroxyacetone phosphate under the influence of aldolase (F) to produce L-sorbose-1-phosphate and it is the latter which is the inhibitory agent. Certain partially purified hexokinase preparations are not affected by glyceraldehyde but are inhibited completely by .0005 M synthetic sorbose-1-phosphate.

The hexokinase reaction is probably not reversible under physiological conditions since ΔF for reaction A is probably about -8000 to -9000 calories. Phosphorylated glucose is probably reconverted to free sugar by the action of a phosphatase on glucose-6-phosphate. It is by this pathway that liver glycogen is converted to blood sugar (Cori, Cori and Schmidt, 1939; Ostern, Herbert and Holmes, 1939; Broh-Kahn, Mirski, Perisutti and Brand, 1948).

Colowick and Kalckar (1943) have demonstrated that hexokinase transfers only one mole of phosphate from ATP to glucose. In the presence of a heat stable enzyme from muscle both high energy phosphate groups can be transferred. The muscle enzyme - myokinase - catalyzes the reversible reaction

$$2\ ADP \longleftrightarrow ATP + AMP.$$

B. PHOSPHOGLUCOMUTASE



Glucose-1-phosphate
"Cori Ester"

Glucose-6-phosphate
"Robison Ester"

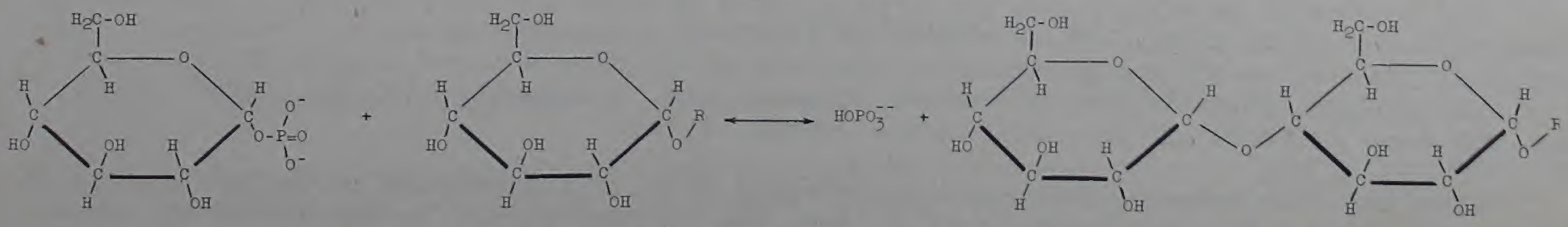
The enzyme which catalyzes the interconversion of the Cori ester and the Robison ester was first studied by Cori, Colowick and Cori (1938a). The crude enzyme is activated by Mg^{++}, Mn^{++} or Co^{++} and exhibits greatest activity at pH 7.5 to 9 (Cori, Colowick and Cori, 1938b). Colowick and Sutherland (1942) reported the above reaction to be at equilibrium when 94.5% of the Cori ester had been converted to glucose-6-phosphate; pH did not influence the point of equilibrium appreciably.

From the work of Meyerhof, Ohlmeyer, Gentner and Maier-Leibnitz (1938) and of Schlamowitz and Greenberg (1947), it can be concluded that the enzyme does not liberate either glucose or phosphate to the surrounding medium. An intramolecular diester, glucose-1,6-monophosphate, has been proposed by Schlamowitz and Greenberg (1947) as the intermediate stage in the interconversion of the two well known glucose monophosphates. These workers state that the proposed intermediate can be constructed with models and shown to be strain-free. However, it seems unlikely that the compound proposed by them is an intermediate in the interconversion of the Cori and Robison esters. The Cori ester has been demonstrated to be of α configuration at carbon atom No. 1 (Cori, Colowick and Cori, 1937; Wolfrom et al., 1941, 1942). A glucose ester containing a single mole of phosphate esterified with both the 6 hydroxyl and the α hemiacetal hydroxyl cannot be drawn on paper or constructed with Fisher-Hirchfelder models. (The formula of the proposed intermediate was drawn by Schlamowitz and Greenberg (1947) as having the β configuration.)

Phosphoglucomutase has been isolated from rabbit muscle extract by Najjar (1948) as a crystalline, electrophoretically homogeneous (at pH 5.0) protein. This pure protein required magnesium ions (.001 M opt.) and cysteine (.01 M opt.) for activity. At 30° and pH 7.5, 100,000 gm. of the enzyme converts 16,800 moles of glucose-1-phosphate to glucose-6-phosphate per minute. The enzyme is sensitive to fluoride ions and it appears that a complex of magnesium, fluoride and organic phosphate is the inhibitory agent (compare the inhibition of enolase by fluoride in section K).

A brief report by Leloir and co-workers (1948) indicates that phosphoglucomutase requires a coenzyme. Should this be confirmed, it would be necessary to conclude that the purified preparation of Najjar contains the coenzyme attached to the protein.

C. PHOSPHORYLASE



Glucose-1-Phosphate
"Cori Ester"

terminal glucose ⟷ phosphate +
residue of glycogen

Glycogen
"Hansen-Bernard
Polysaccharide"

Until 1936, the mechanism of the glycogen ⟷ glucose interconversion was entirely unknown. Some workers believed that ATP was required to initiate hexose phosphate formation from glycogen while others thought that liver glycogen was converted to blood glucose by amylase. Both of these contentions have been disproved. It is now known that glucose-1-phosphate is an intermediate in the synthesis and breakdown of glycogen in both animal and plant cells.

The clue to the elucidation of this mechanism was the finding by Cori and Cori (1935) working with intact frogs and by Parnas and Baranowski (1935) working with muscle extracts that inorganic phosphate is esterified when glycogen is broken down to produce a hexose monophosphate. The hexose monophosphate which accumulates is a mixture of glucose- and fructose-6-phosphates ("Embden ester") (Ostern, Guthke and Terszakowec, 1936). It was just prior to these investigations that the Coris, who had for many years been studying carbohydrate metabolism and especially the hexose phosphates, changed their investigative approach from the intact animal to tissue extracts. Their first paper describing work with minced frog muscle (Cori and Cori, 1936) disclosed the fundamental fact that during the uptake of inorganic phosphate a new, non-reducing ester is formed from glycogen. This ester had the properties of an aldose-1-phosphate and was demonstrated to be a precursor of glucose-6-phosphate. Cori, Colowick and Cori (1937) proved the structure of the new ester by its synthesis (cf. also Wolfrom et al., 1941-42).

The reversibility of reaction C in yeast extracts was demonstrated by Schäffner and Specht (1938, 39) and Kiessling (1939) and in muscle, liver, heart and brain extracts by Cori, Schmidt and Cori (1939). The equilibrium of the reaction depends upon pH (Hanes, 1940; Cori and Cori, 1940). According to Hanes and Maskell (1942), the equilibrium ratio of the mono or divalent salts of glucose-1-phosphate and orthophosphate remain constant between pH 6 and pH 7 and can be expressed as $\frac{HPO_4^{--}}{\text{Glucose-1-}PO_4^{--}} = 2.2$.

Muscle phosphorylase was first obtained in crystalline form by Green, Cori and Cori (1942). The enzyme is an euglobulin with a molecular weight of from 340,000 to 400,000 and constitutes about two percent of the protein in a water extract of ground rabbit muscle (Green, Cori and Oncley, 1943). This purified enzyme will synthesize glucose-1-phosphate from either starch or glycogen and inorganic phosphate. For the reverse reaction, however, it is not sufficient merely to add the substrate to the enzyme. To obtain polysaccharide synthesis from glucose-1-phosphate a small amount of glycogen "primer" must be present.

In the early studies on muscle phosphorylase, aged, dialyzed extracts of rabbit muscle were usually employed. Such extracts show very little phosphorylase activity unless adenylic acid is added along with glycogen and inorganic phosphate. Inosinic acid activates phosphorylase slightly while adenosine tri-phosphate is practically ineffective (Cori, Colowick and Cori (1938a). Crystalline phosphorylase prepared rapidly in the cold from anesthetized rabbits' muscle (Green, Cori and Oncley, 1943) exhibits 65% of its maximum activity without added adenylic acid. Maximum activity is obtained with about 10^{-5} M adenylic acid. This preparation has been designated phosphorylase a. It is converted to phosphorylase b, which requires adenylic acid, by the action of PR (prosthetic group removing) enzymes (Cori and Cori, 1945b) which are present in a variety of tissues. It could not be demonstrated that the substance removed from phosphorylase a (by PR enzyme) was actually adenylic acid. The conversion of phosphorylase a to b occurs also in muscle during contraction to fatigue (Cori, 1945) and apparently the reverse occurs during rest. Mrs. Cori has postulated that "The temporary inactivation of phosphorylase a by enzymatic removal of its prosthetic group may represent a regulatory mechanism which would prevent the exhaustion of glycogen stores in fatigue."

The b form of phosphorylase was crystallized by Cori and Cori (1945a). With added adenylic acid it is about 90% as active as crystalline phosphorylase a. Studies of the physical properties of phosphorylase b indicate it to have a lower electrophoretic mobility than phosphorylase a; the diffusion constant and, presumably, the molecular weight, are the same as those of phosphorylase a (Green, 1945).

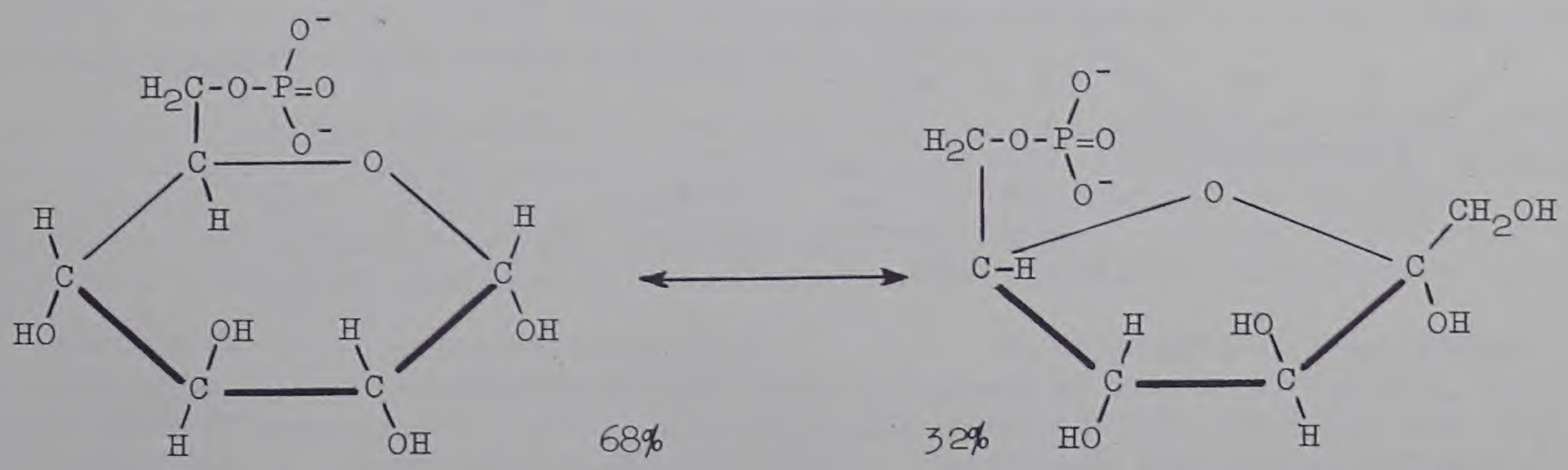
When purified muscle phosphorylase converts glucose-1-phosphate to polysaccharide in the presence of a minimal quantity of primer, the polymer formed is not glycogen. It

stains blue with iodine and appears to be a linear polysaccharide of the amylose type containing glucose residues in 1:4 linkage (Hassid, Cori and McCready, 1943). Heart and liver phosphorylase preparations, however, contain an additional enzyme which produces 1:6 linkages. When such preparations are added to muscle phosphorylase, the combined action of the two enzymes produces true glycogen from glucose-1-phosphate.

Plant phosphorylase does not require additions of adenylic acid. It differs also in that comparatively simple oligosaccharides can serve as the "primer", while muscle phosphorylase requires a large, branched-chain polysaccharide such as amylopectin or glycogen. Proehl and Day (1946) estimated that the activator for potato phosphorylase need be no larger than 6 to 7 glucose residues per molecule. Swanson and Cori (1948) found that polysaccharide fragments containing only 4 to 5 glucose units would activate potato phosphorylase. The latter workers demonstrated that if these fragments were derived from amylopectin type polysaccharide, the primer must have end groups at least "3 to 4 glucose units removed from a branch point".

Bourne and Peat (1945) have obtained two types of phosphorylase from potato juice. One of these (P enzyme) converts glucose-1-phosphate to a polysaccharide which stains blue with iodine. The other (Q enzyme) apparently makes amylopectin, for its product stains red. The Q enzyme can rearrange the glucose units of amylose to give a product resembling amylopectin (Bourne, Macey and Peat, 1945).

D. PHOSPHOHEXOISOMERASE



Glucose-6-Phosphate
"Robison ester"

Fructose-6-Phosphate
"Neuberg ester"

The enzymatic transformation of fructose-6-phosphate to glucose-6-phosphate was first described by Lohmann (1933). While studying the dephosphorylation of HDP by dialyzed frog muscle extracts, he observed that the product was not the Neuberg ester, as expected, but a mixture of aldohexose monophosphate and ketohexose monophosphate. He then found that the enzyme which formed this mixture (Embden ester) from either the Neuberg ester or from aldohexosemonophosphate was present in extracts of many different organs. It has since been demonstrated by Hanes (1940) in plants.

Gottschalk (1947), on the basis of earlier work, considers glucose-6-phosphate and mannose-6-phosphate to be converted to the Neuberg ester by separate enzymes in yeast. Comparable studies have not yet been made with animal tissues, but it is known that mannose is metabolized by a great variety of animal cell preparations.

E. PHOSPHOHEXOKINASE

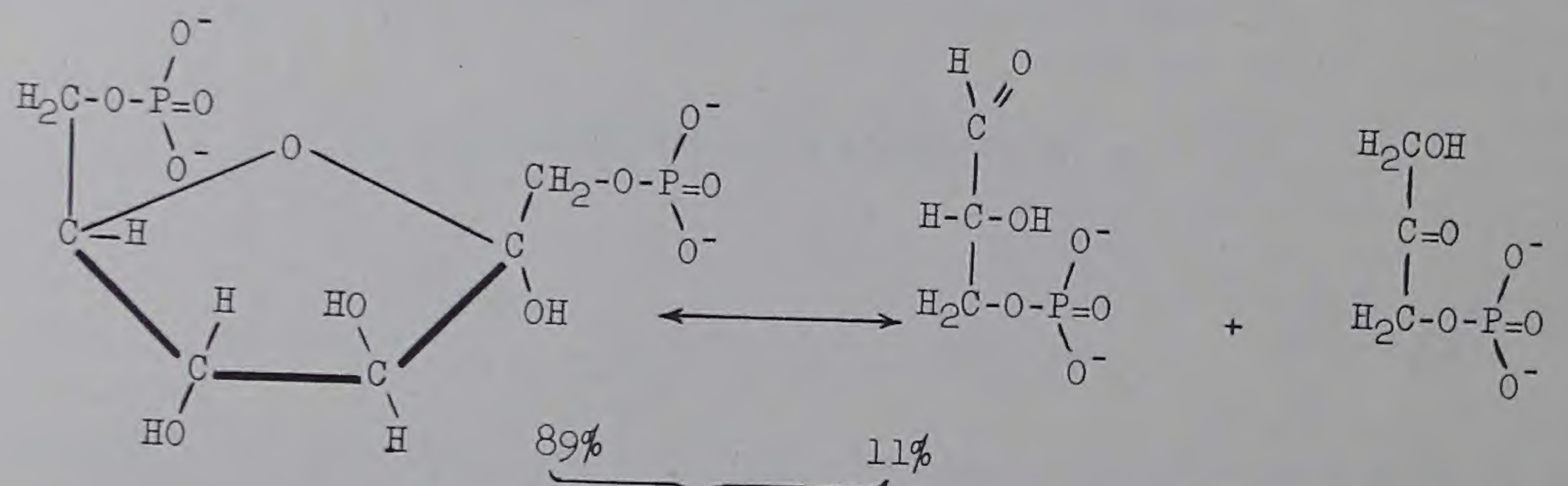
Fructose-6-phosphate + ATP ⟶ Fructose 1,6 diphosphate + ADP

The fact that adenosine triphosphate plays an important role in <u>initiating</u> carbohydrate metabolism was originally interpreted to mean that ATP phosphorylated glycogen.

Following the demonstration that inorganic phosphate splits glycogen to HMP, Ostern, Guthke and Terszakowec (1936) found that the HMP was phosphorylated to HDP by adenosine triphosphate. The enzyme which carries out this reaction has not been studied extensively. Cori and Slein (1947) have found that phosphohexokinase preparations from muscle will phosphorylate either the 1- or 6-phosphoric esters of fructose to HDP.

It seems doubtful that HDP is converted to HMP during glycogen synthesis from lactate by a reversal of reaction E. The 3,000 calorie bond between phosphate and fructose is much more stable than the pyrophosphate linkage of ATP. In order to obtain any appreciable rate of glycogen formation, it would seem that the conversion probably is made by a phosphatase with the resultant loss of the fructose-phosphate bond energy. Gomori (1943) has described a Mg^{++} activated phosphatase which is apparently specific for hexosediphosphate. The enzyme removed only one phosphate group from HDP but unfortunately the product was not characterized.

F. ALDOLASE



Fructose-1,6-diphosphate — "Harden-Young Ester"

D-Glyceraldehyde-3-phosphate — "Fischer-Baer Ester"

Dihydroxyacetone phosphate — "Kiessling Ester"

The elucidation of the glycolytic pathway by which the six-carbon sugars are converted to the three-carbon chain of lactic acid was a problem which vexed biochemists for many years. Until 1932 methyl glyoxal was considered by many to be the immediate precursor of lactic acid. Here was a compound which could be isolated from fermentation or glycolysis mixtures, and an enzyme discovered many years earlier was known to convert methyl glyoxal to lactic acid. However, in 1932 Lohmann demonstrated that this enzyme -- glyoxalase -- required glutathione as a coenzyme and that muscle extracts freed of glutathione but fortified with other cofactors could convert glycogen to lactic acid even though methyl glyoxal was not so converted. In the same year Fischer and Baer (1932) reported the synthesis of DL-glyceraldehyde-3-phosphate, a hypothetical intermediate in Ohle's (1931) scheme of carbohydrate breakdown. It was soon found that this synthetic ester was fermented to the extent of 50% by yeast zymase (Smythe and Gerischer, 1933) leaving the levo rotatory component. Embden and Jost (1934) found it was converted to lactic acid by muscle preparations and Meyerhof and Kiessling (1933a) found it to produce phosphoglyceric acid in the presence of fluoride.

Following these findings, Embden postulated a complete scheme of carbohydrate breakdown which has proved to be almost exactly in accord with present day knowledge. (As early as 1913 Embden and co-workers had postulated the reversible cleavage of ketoses into glyceraldehyde and dihydroxyacetone in the metabolism of carbohydrate.)

The enzymatic conversion of HDP to triose phosphate was established by Meyerhof and Lohmann (1934). They not only isolated dihydroxyacetone phosphate but demonstrated that hexose diphosphate could be formed from it. This demonstration was facilitated by their discovery that the above equilibrium is greatly influenced by temperature. At 60° almost all of the HDP is converted to triose phosphate while at 0° the reverse is true. The reaction was formulated as:

HDP ⟷ 2 Dihydroxyacetone phosphate

since the isomerase in their enzyme preparation rapidly converted D-glyceraldehyde-3-phosphate to dihydroxyacetone phosphate (see Section G).

From the work of Meyerhof, Lohmann and Schuster (1936) we may conclude that aldolase is specific for the dihydroxyacetone phosphate component of reaction F but can utilize a wide variety of aldehydes. For example, if acetaldehyde and HDP are added to dialyzed muscle extract the HDP gradually disappears and a new ester, methyl tetrose phosphate is formed. When, instead, D-glyceraldehyde is added with HDP, the final product is fructose-1-phosphate indicating that the free aldehyde condenses with dihydroxyacetone phosphate. DL-glyceraldehyde was found to react with dihydroxyacetone phosphate to produce an equimolar mixture of D-fructose-1-phosphate and L-sorbose-1-phosphate indicating that both isomers are utilized. This condensation undoubtedly explains the early finding by Embden, Schmitz and Wittenberg (1913) that perfused dog liver converted DL-glyceraldehyde to a mixture of D-glucose and L-sorbose (at that date termed d-sorbose). Aldolase is not completely unspecific with regard to the aldehyde component of reaction F for the author has found (see Lardy and Ziegler, 1945) that purified preparations of aldolase and isomerase do not condense L-glyceraldehyde-3-phosphate to a HDP.

A consideration of the products formed when various aldehydes were mixed with HDP in the presence of muscle extracts indicated that the aldolase reaction should be formulated as equation F above. Proof for this contention was later obtained by Meyerhof (1938) when, by trapping the products of HDP cleavage with hydrazine, it was found that one mole of glyceraldehyde phosphate and one of dihydroxyacetone phosphate were formed from each mole of HDP split.

Purified preparations of aldolase have been obtained by Herbert, Gordon, Subrahmanyan and D. Green (1940), Engelhardt (1942), Meyerhof and Beck (1944), Warburg and Christian (1943) and by Taylor, A. Green and G. Cori (1948). Warburg's and Christian's rat muscle preparation is the most active reported. Crystalline rabbit muscle aldolase is prepared by Taylor et al. as considerably less active (turnover number 1700 moles HDP/min./150,000 gm. at 30°) even though it appears to be electrophoretically homogeneous over a wide pH range. Diffusion constants (Taylor et al.) and amino acid analyses (Velick and Ronzoni, 1948) indicate a molecular weight of about 150,000 for rabbit muscle aldolase.

Purified aldolase shows optimum activity at pH 9. The equilibrium data cited above are taken from Herbert et al. (1940) but are applicable only for the initial substrate concentration given.

Purified aldolase from muscle is extremely sensitive to traces of heavy metals (Ag^{+}, Cu^{++}, Zn^{++}) as well as to I_2. Warburg and Christian (1943) find yeast aldolase to be inhibited by pyrophosphate, dipyridyl, cysteine and GSH indicating that the enzyme has a dissociable heavy metal activator. Yeast aldolase inactivated by the above complex formers is reactivated by Fe^{++} or Zn^{++}.

A method has been devised by Allen and Bourne (1943) for the microscopic detection of aldolase in tissue slices. The aldolase of a plant tissue (pea seeds) has been partially purified by Stumpf (1948).

It would seem that the ability of aldolase to condense a variety of aldehydes with dihydroxyacetone phosphate offers a possible pathway for the formation of a wide variety of naturally occurring compounds in addition to hexose diphosphate.

G. TRIOSEPHOSPHATE ISOMERASE

Dihydroxyacetone phosphate ⟷ D-Glyceraldehyde-3-phosphate
96% 4%

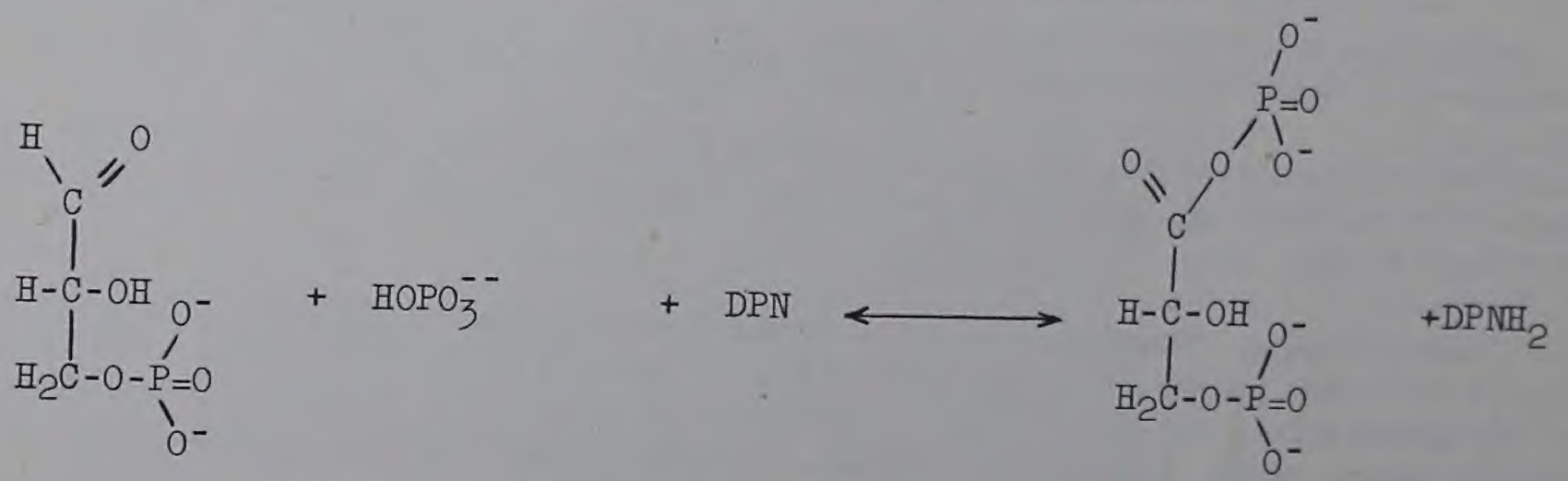
The enzyme which catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate is a discovery of Meyerhof and Kiessling (1935a).

As a result of the action of aldolase on HDP one mole of each of the triose phosphates is produced. In the presence of a muscle extract the aldose form is rapidly converted to the ketose until an equilibrium of 4% aldotriosephosphate:96% ketotriosephosphate is reached. It is this fact which originally led Meyerhof and Lohmann (1934) to believe that two molecules of dihydroxyacetone phosphate are produced from each mole of HDP. When the aldolase reaction was carried out in the presence of carbonyl reagents like hydrazine or sulfite, half of the product was glyceraldehyde phosphate (Meyerhof, 1938).

The point of equilibrium of the reaction has been fairly accurately established by Meyerhof and Junowicz-Kocholaty (1943) despite the considerable difficulty of determining one triose phosphate in the presence of the other. If the equilibrium mixture is treated with iodine in a mildly alkaline solution, the aldose component is oxidized to D-3-phosphoglyceric acid. This ester may be estimated by measuring its rotation in the presence of molybdate ($[\alpha]_D = -740^\circ$) as described by Meyerhof and Schulz (1938). Alternatively, the ketotriose ester remaining after iodine oxidation may be determined by the conventional alkaline hydrolysis procedure (1N NaOH, 20 min. at room temperature) and measurement of inorganic phosphate liberated. However, it is difficult to obtain complete oxidation of the aldose phosphate without destroying some of the ketonic ester and precautions must be taken to avoid excess alkalinity which destroys both esters (Fischer and Baer, 1932).

Purification of the enzyme from rabbit muscle has been achieved to the point where the "turnover number" (moles substrate transformed to product by 100,000 gms of enzyme protein per minute at 38°) is approximately 1,000,000 (Meyerhof and Beck, 1944). It is thus one of the most active enzymes known. Assuming that Meyerhof and Beck have obtained a nearly pure enzyme, isomerase represents about 4% of the water extractable proteins of rabbit muscle. Purified triose phosphate isomerase preparations have no phosphohexoisomerase activity; they are inactivated by dialysis.

H. TRIOSEPHOSPHATE DEHYDROGENASE



D-Glyceraldehyde-3-phosphate — orthophosphate — 1,3-Diphosphoglycerate "Negelein-Bromel Ester"

The uptake of inorganic phosphate during fermentation had been well established by Harden but the mechanism of this fixation remained obscure until about 1936 when it was found that there are two sites of orthophosphate uptake. One has been discussed under C above. Light was thrown on the second by Schäffner and Berl (1936) when they demonstrated that the esterification depended upon a simultaneous exothermic oxidation-reduction reaction. They found this step to be promoted by HDP and not abolished by fluoride. The latter observation set the new process apart from the conversion of phosphopyruvate to pyruvate and ATP.

D. Needham and Pillai (1937) and Meyerhof (1937) found the phosphate uptake to occur during the reaction: pyruvate + triose phosphate + adenylic acid + orthophosphate ⟶ lactate + phosphoglycerate + adenylpyrophosphate. It was then shown by Green, Needham and Dewan (1937) that the triose oxidation may be reversed with either lactate or malate as the electron donor. The reversibility was confirmed by Meyerhof, Ohlmeyer and Mohle (1938) who also demonstrated the necessity for ATP in bringing about the reduction of 3-phosphoglycerate to triose phosphate.

In 1939 Warburg and Christian isolated triose phosphate dehydrogenase in a crystalline state from yeast and used this preparation to study the mechanism of the oxidative step. Oxidation of triose phosphate and reduction of DPN were found to occur only in the presence of inorganic phosphate. The oxidation product was isolated by Negelein and Brömel (1939) and shown to be a diphosphoglyceric acid. The chemical properties of this labile ester indicated it to be an acid anhydride of phosphoric and 3 phosphoglyceric acids.

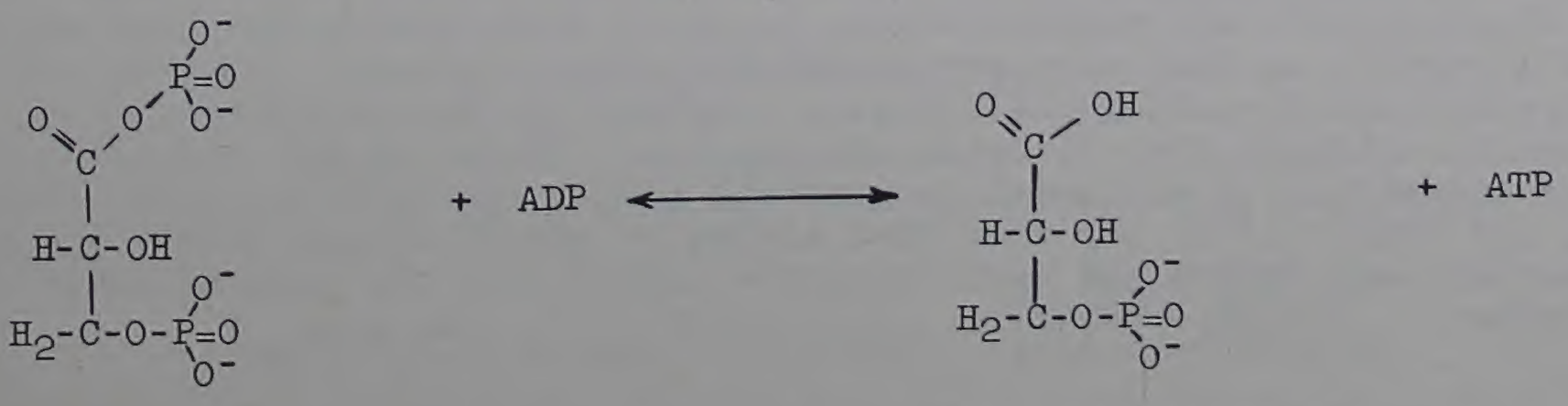
It is known (see Lipmann 1941; Kalckar 1941) that the oxidation of aldehydes to carboxylic acids is a strongly exothermic reaction. Resonance of the carboxyl group contributes to the stability of the acid. For this reason acids are not easily reduced to aldehydes. In the biological oxidation of glyceraldehyde-3-phosphate, however, a stable product is not produced. The "opposing resonance" of the acid anhydride linkage in diphosphoglycerate makes a labile, "high-energy" compound which can be reduced readily to the aldehyde. The transfer of the high-energy phosphate to ADP to produce ATP and the stable 3-phosphoglycerate is discussed in section I.

Triose phosphate dehydrogenase has also been isolated from rabbit muscle (Cori, Slein and Cori, 1945; Caputto and Dixon, 1945). The purified enzyme from muscle is inactivated by storage, presumably by an oxidative reaction. The inactive "oxidized" form is reactivated by cysteine or GSH. In confirmation of earlier work by Meyerhof and Kiessling (1933a and b) and Rapkine (1938), the enzyme is inactivated by iodoacetate. The reaction with iodoacetate is not instantaneous and is not reversed by cysteine.

When the reaction components are limited one at a time, the dehydrogenase exhibits half maximum activity when the concentration of glyceraldehyde phosphate is 5.1×10^{-5} M or when the concentration of DPN is 3.9×10^{-5} M (Cori, Slein and Cori, 1948). At pH 8.6 and 27°, 100,000 grams of the crystalline enzyme will reduce 6700 moles of DPN per minute. At pH 7.1 the activity is only about 15% as great. The enzyme oxidizes free glyceraldehyde but only about 1/300th as rapidly as the phosphorylated aldehyde. Caputto and Dixon (1945) indicated that inorganic phosphate was necessary for the oxidation of both the free and phosphorylated glyceraldehyde.

In spite of intensive study by Meyerhof and his collaborators, (Meyerhof and Junowicz-Kocholaty, 1943, Drabkin and Meyerhof, 1945) the mechanism by which orthophosphate is fixed during triose oxidation is still obscure. It is believed that phosphate adds to the aldehyde just as water does to form aldehyde hydrate. Oxidation of such an aldehyde phosphate $-C(OH)H-OPO_3H_2$ would then leave an acyl phosphate $-C(=O)-OPO_3H_2$ Other mechanisms have been proposed without experimental support.

I. ATP - PHOSPHOGLYCERIC TRANSPHOSPHORYLASE



1,3-Diphosphoglycerate

3-Phosphoglycerate
"Nilsson Ester"

The enzyme catalyzing the above equilibrium has been isolated from yeast in crystalline form by Bücher (1947). Purification of the enzyme from Lebedev juice was followed by a spectrophotometric method which was likewise employed for an intensive study of the above reaction with the purified enzyme. The dissociation constants for the enzyme and each of the reaction constituents were determined with the following results:

Complex between enzyme and	Dissociation constant
ADP	2×10^{-7} Mole/ml
ATP	1.1×10^{-7} "
D- 1,3 diphosphoglycerate	1.8×10^{-9} "
D- 3 phosphoglycerate	2×10^{-7} "
Mg^{++}	2.5×10^{-7} "

The forward reaction was catalyzed at a rate of 320,000 moles per minute per 100,000 g protein. The protein, which comprises about 0.2% of the weight of fresh yeast, has an unusually small absorption at 280 mu indicating a low content of tryptophan and tyrosine.

The crystalline protein was ash free when dialyzed but required the addition of Mg or Mn ions for activity. While no requirement for either of these ions could be demonstrated with dialyzed muscle extract (Lardy and Ziegler, 1945), it must be recognized that sufficient divalent cation might be retained by the protein to permit the reaction to proceed at a rapid rate (see section L for a discussion of the retention of K^+ by these extracts).

Bücher determined the equilibrium of the above reaction to be

$$\frac{[\text{ATP}]\ [\text{Phosphoglycerate}]}{[\text{ADP}]\ [\text{Diphosphoglycerate}]} = 3.3 \times 10^3$$

at neutrality. The equilibrium was not markedly dependent upon pH.

J. PHOSPHOGLYCEROMUTASE



D-3-Phosphoglycerate ⟷ D-2-Phosphoglycerate "Meyerhof-Kiessling Ester"

In 1930 Lohmann demonstrated that a stable phosphoric ester accumulates in fluoride poisoned muscle extract. Nilsson (1930) isolated the barium salt of the ester which accumulated in fluoride poisoned yeast extracts to which acetaldehyde had been added and predicted its structure as that of a monophosphoglycerate. Embden, Deuticke and Kraft (1933) and Meyerhof and Kiessling (1933b) also obtained the monophosphoric ester from glycolyzing muscle extracts when fluoride was present. Embden et al. demonstrated that this ester was enzymatically converted to pyruvate and inorganic phosphate. About this time, Neuberg and Kobel (1933) reported that synthetic monophosphoglycerate was metabolized in the same manner, and Embden and Deuticke (1934) identified the natural ester as monophosphoglycerate.

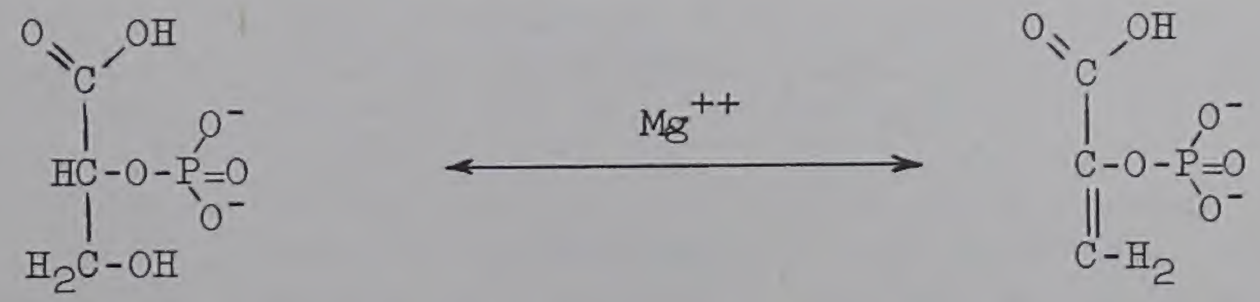
In 1935 Meyerhof and Kiessling reported that both muscle and yeast extracts catalyzed the transformation of 3-Phosphoglycerate into a new intermediate 2-Phosphoglycerate and that the reaction was reversible. At that time they reported the equilibrium to vary considerably with temperature. Later Meyerhof and Schulz (1938) applied their more accurate method for determining these esters (rotation in the presence of molybdate) and observed the following equilibria:

Temperature (°C)	% 2-Phosphoglycerate
0	8.5
20	9.5
37	11.2
60	11.6

When reaction (J) is carried out in the presence of inorganic phosphate containing P^{32}, no radioactive P is found in the product (Meyerhof, Ohlmeyer, Gentner and Maier-Leibnitz, 1938), hence the phosphorous atoms originally on the carbon chains remain attached.

It is remarkable that a metabolic function has never been found for the 2,3-diphospho-D-glycerate discovered in erythrocytes by Greenwald (1925) many years before the monophosphoglycerates were known. This diphosphoglycerate can be converted to pyruvate by a variety of cell extracts (cf. Neuberg, Schuchardt and Vercellone, 1934; Lennerstrand, 1939), but the pathway by which it arises and the detailed mechanism of its breakdown have yet to be discovered.

K. ENOLASE



D-2-Phosphoglycerate

Phosphoenolpyruvate
"Lohmann-Meyerhof Ester"

The phosphoric ester of enol-pyruvic acid was discovered by Lohmann and Meyerhof (1934) while studying the metabolism of phosphoglycerate by dialyzed muscle extracts. It was later synthesized by Kiessling (1936). The enzymatic conversion of 2-phosphoglycerate to phosphopyruvate was studied by Meyerhof and Kiessling (1935b) who found that fluoride inhibited the formation of phosphopyruvate from 2-phosphoglycerate but did not inhibit the formation of the latter from 3-phosphoglycerate. Thus they established the site of fluoride inhibition of glycolysis which had been demonstrated earlier by Embden and Haymann (1924) and which had been partially located by Lohmann and Meyerhof (1934). The mechanism by which fluoride exerts its inhibition was disclosed by Warburg and Christian (1942) in a study of the pure enolase which they prepared from yeast as a crystalline mercury complex. The purified enzyme when freed of mercury was active when either Mg^{++}, Mn^{++} or Zn^{++} was added. Inhibition of the enzyme by fluoride results from the formation of a magnesium-fluoride-phosphate complex which apparently combines with the enzyme and thereby displaces the normal activator - Mg^{++}. Warburg and Christian found that the inhibition could be expressed as:

$$C_{Mg^{++}} \times C^2_{fluoride} \times C_{phosphate} \times \frac{\text{residual action}}{\text{inhibited action}} = K$$

at pH 7.34 and 20°, $K = 3.2 \times 10^{-2}$

Manganese does not form such an inhibitory complex and hence fluoride is less effective with Mn^{++} rather than Mg^{++} as the activator (Warburg and Christian, 1942; Utter and Werkman, 1942; Massart and Dufait, 1942). These results explain earlier observations (see Meyerhof and Schulz, 1938) that fluoride does not effectively block the enolase reaction unless inorganic phosphate is present.

According to Warburg and Christian pure enolase activated with Mg^{++} converts 9900 moles of substrate per minute per 100,000 gms of enzyme at 20° and pH 7.34. Half maximum activity was obtained when 2-phosphoglycerate was present at 1.5×10^{-4} M. Bücher (1943) determined the molecular weight of enolase to be 62,000. Both the crystalline mercury salt of enolase and the mercury-free protein which had been fully activated with Mg^{++} were found to be combined with their respective metals in a 1:1 molar ratio. An interesting observation of Bücher's is that the enzyme is apparently dissociated into catalytically inactive particles of smaller size by dialysis at pH 5. The original particle size and biological activity were restored by adding salt.

The equilibrium constant of reaction K is 1.43 at pH 7.34 and 20° according to Warburg and Christian (1942). Meyerhof and Schulz (1938) and Utter and Werkman (1942) obtained lower values. Since the equilibrium constant of the reaction is very close to 1, the free energy of the reaction must be near zero. In spite of this fact, 2-phosphoglycerate has a stable low-energy ester phosphate linkage while phosphopyruvate has a high-energy enol phosphate group which is capable of being transferred to ADP to form ATP. This is explained by Lipmann (1941) as resulting from a concentration of a higher fraction of the total energy of the molecule in the phosphate linkage in enol-pyruvic acid.

L. ATP - PHOSPHOPYRUVATE TRANSPHOSPHORYLASE

$$\text{Phosphoenolpyruvate} + \text{ADP} \xleftrightarrow[Mg^{++}]{K^{+}} \text{Pyruvate} + \text{ATP}$$

The enzymatic transfer of phosphate from phosphopyruvate to the adenylic system was first observed by Parnas, Ostern and Mann (1934) while studying the metabolism of phosphoglycerate. The fact that phosphopyruvate is an intermediate in this transfer was established by Lohmann and Meyerhof (1934). The transphosphorylating enzyme from muscle requires both K (Boyer, Lardy and Phillips, 1942, 1943) and Mg (Lohmann and Meyerhof, 1934) ions for activity. Ammonium ions can replace the function of K^+. Sodium ion antagonizes the function of K^+ in that it depresses the activity of the enzyme in the presence of limiting K^+; this depression may be overcome partially by further additions of K^+. Calcium inhibits the transfer of phosphate from phosphopyruvate to ADP (Boyer et al., 1942, 1943). Thus calcium and sodium on one hand and potassium on the other seem to regulate the activity of this transphosphorylase in a manner strikingly similar to their effect on the perfused heart preparation.

According to Muntz (1947) the transphosphorylating enzyme from yeast does not require K^+ (confirmed by Betheil, 1947).

For many years reaction L was considered by numerous writers to be irreversible following the report of Meyerhof, Ohlmeyer, Gentner and Maier-Leibnitz (1938) that radioactive phosphorus in ATP did not exchange with the phosphorus in phosphopyruvate in the presence of a dialyzed muscle extract. Since all enzymatic reactions must be reversible and since Lipmann (1941) had calculated the bond energy of phosphate-enolpyruvate to be only somewhat greater than that of the pyrophosphate linkage the subject was reinvestigated. It was found (Lardy and Ziegler, 1945) that the exchange of P^{32} did occur between phosphopyruvate and ATP and that phosphopyruvate could be synthesized directly from pyruvate in a system where ATP was constantly being regenerated. The exchange reaction was hastened by the addition of K^+ but occurred at an appreciable rate even in the absence of added K^+. Well dialyzed muscle extracts still contain potassium in appreciable quantities and it is probable that this residual potassium activates the transphosphorylase.

Kubowitz and Ott (1944) have isolated ATP-phosphopyruvate transphosphorylase from human muscle. The crystalline enzyme was only slightly soluble in water but readily

soluble in neutral or weakly basic phosphate buffers. At 20° and pH 6.77, 100,000 gm of protein transfers 6,000 moles of high-energy phosphate from phosphopyruvate to ADP.

M. LACTIC DEHYDROGENASE

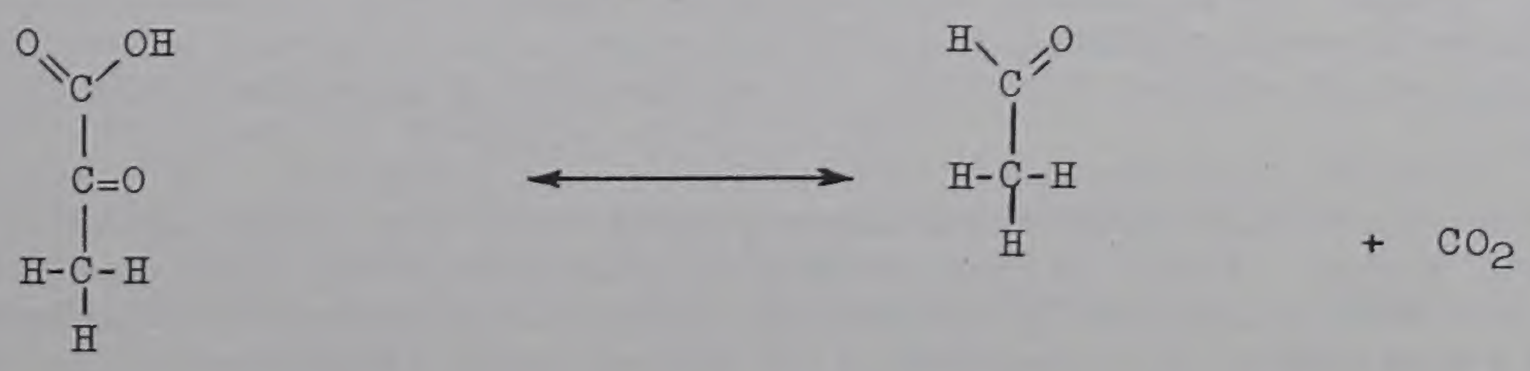


Pyruvate L-Lactate

The lactic dehydrogenase of animal tissues has been prepared in pure form from more different sources than have most other dehydrogenases. The dehydrogenase transports electrons between coenzyme I and lactic or pyruvic acids. Coenzyme II can replace DPN for the dehydrogenase obtained from ox heart, rabbit muscle or pigeon liver but is only 1/200 to 1/100 as active as DPN (Mehler et al., 1948). The lactic dehydrogenase of muscle was first studied by Meyerhof (1919), and Szent-Györgyi (1925) discovered the necessity of the coenzyme. The enzyme specifically forms and dehydrogenates lactic acid of L configuration (Meyerhof and Lohmann, 1926).

Lactic dehydrogenase was first prepared in crystalline form from beef heart by Straub (1940). The crystalline enzyme has since been obtained from rat muscle and Jensen sarcoma by Kubowitz and Ott (1943). The enzymes from the normal and neoplastic rat tissues were apparently identical. These same workers (Kubowitz and Ott, 1944) purified the enzyme from human muscle until it possessed the same degree of activity as their crystalline preparation from rat muscle (100,000 gm protein reduced 22,500 moles of pyruvate per minute at 20° and pH 7.4). However, they were unable to continue their work and a crystalline preparation was not obtained.

N. PYRUVATE DECARBOXYLASE



Pyruvate Acetaldehyde

The conversion of pyruvate to two-carbon compounds in animal tissues falls in the province of the following chapter, in yeast however this step is still in the fermentative pathway. The role of pyruvate in yeast fermentation was predicted by Neubauer and Fromherz (1910) and established by Neuberg and Karczag (1911). The enzyme which converts pyruvate to acetaldehyde and CO_2 has been called "carboxylase"; the term pyruvate decarboxylase is used here to differentiate this enzyme from those which decarboxylate the other numerous α and β keto acids. Auhagen (1931-32) discovered that a heat stable coenzyme is required by this carboxylase and that it may be washed from the yeast protein with alkaline phosphate. The coenzyme was isolated and identified as the pyrophosphoric ester of thiamine by Lohmann and Schuster (1937).

The pyruvate decarboxylase of brewers yeast has been purified by Green, Herbert and Subrahmanyan (1940, 1941) and by Kubowitz and Lüttgens (1941). The purest preparations

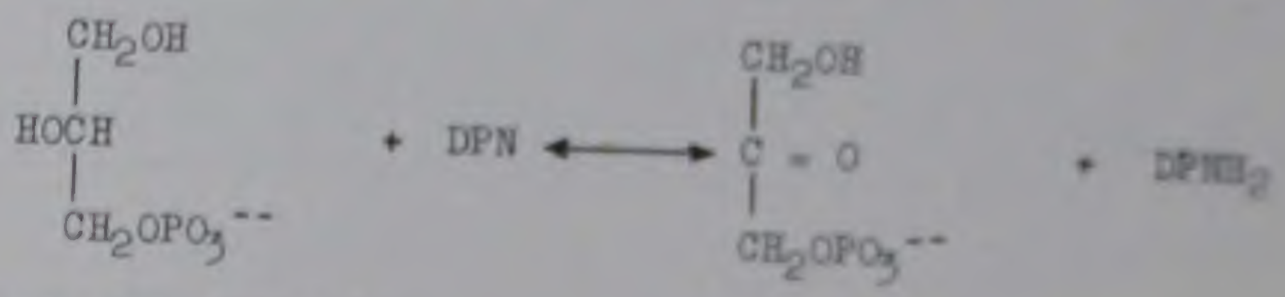
contain magnesium and thiamine pyrophosphate in a 1 to 1 molar ratio and apparently there are 2 moles of each per mole of enzyme whose molecular weight is about 141,000 according to Melnick and Stern (1940).

O. ALCOHOL DEHYDROGENASE

$$CH_3CH_2OH + DPN \longleftrightarrow CH_3CHO + DPNH_2$$

Alcohol dehydrogenase from yeast is not entirely specific for ethanol for it will attack a variety of primary and secondary alcohols. The equilibrium of the above reaction is far toward the left and thus serves to oxidize triose phosphate under anaerobic conditions. Negelein and Wulff (1936, 1937) have obtained the enzyme in crystalline form from maceration juice of brewers yeast by a procedure which involved heating to 56° to precipitate inert proteins, fractional precipitations with acetone, methanol, ethanol and finally with ammonium sulfate when the enzyme precipitates in crystalline form.

Q. α GLYCEROPHOSPHATE DEHYDROGENASE



L-α Glycerophosphate
"Karrer Ester"

Dihydroxyacetonephosphate

The enzyme which reduces dihydroxyacetone phosphate to α-glycerophosphate is of importance for several reasons. During the "induction phase" of fermentation, before any pyruvate accumulates, dihydroxyacetone phosphate may accept electrons from reduced coenzyme I. This permits a slow oxidation of triose phosphate and eventually sufficient pyruvate and acetaldehyde are formed to permit the latter to accept the electrons. In the "glycerol fermentation", agents are added which combine with pyruvate and acetaldehyde and thus "induction phase" fermentation continues throughout the fermentation period. Phosphatases hydrolyze the accumulated α-glycerophosphate to liberate glycerol. In animal tissues the α-glycerophosphate required for synthesizing phospholipids is produced by the above reaction.

von Euler's school has been particularly active in the study of this dehydrogenase. Adler, Euler and Hughes (1938) separated the enzyme from most other dehydrogenases which accompany it in tissue extracts. β -glycerophosphate and D-α-glycerophosphate are not oxidized. The requirement for coenzyme I is one of many characteristics which differentiate this enzyme from the insoluble α-glycerophosphate dehydrogenase studied by Green (1936).

OPTICAL CONFIGURATION OF THE THREE CARBON INTERMEDIATES IN CARBOHYDRATE METABOLISM

Our knowledge of the optical configuration of the naturally occurring triose phosphates, phosphoglycerates, α-glycerophosphate, phospholipids and glycerol ethers is based on the fundamental work of H. O. L. Fischer and E. Baer (1937, 1941).

In 1926 Karrer and Salomon demonstrated that the α-glycerophosphate in phosphatides is present in only one enantiomorphic form. An unequivocal synthesis of optically active α-glycerophosphates (Fischer and Baer, 1937; Baer and Fischer, 1939) demonstrated that the naturally occurring ester is of L configuration if the carbon atom containing the free primary hydroxyl group is designated number 1. Kiessling and Schuster (1938) then oxi-

dized, with bromine, the natural α-glycerophosphate isolated from yeast and found that the L-3-phosphoglyceric acid produced was dextrorotatory and was not metabolized. Since the natural 3-phosphoglyceric acid is levorotatory it must belong to the D series and the glyceraldehyde-3-phosphate which is its precursor must likewise have the D configuration.

Prior to this time it had been assumed that α-glycerophosphate arose by reduction of glyceraldehyde-3-phosphate (cf. Baldwin, 1947, p. 127, 315, 324, 349 e.g.). In view of their new findings, Fischer and Baer (1937; Baer and Fischer, 1939) proposed that dihydroxyacetone phosphate was the immediate precursor of L-α-glycerophosphate and subsequent work has borne out their contention.

SUMMARY

Carbohydrate breakdown by way of the scheme shown in Figure 1 provides energy to the living cell under anaerobic conditions. For each mole of hexose metabolized, an expenditure of two moles of high energy phosphate is required to prepare the hexose molecule for its cleavage. Four moles of high energy phosphate are returned to the adenylic system per mole of hexose converted to lactate or ethanol. Thus a net gain of two moles of utilizable high-energy phosphate is realized. This is a comparatively small amount of the free energy in the glucose molecule. Far more energy is liberated during the oxidation of the glycolytic end products as will be brought out in the chapter which follows.

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Chapter X

THE CITRIC ACID CYCLE AND THE CYCLOPHORASE SYSTEM

by

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THE CITRIC ACID CYCLE AND THE CYCLOPHORASE SYSTEM

The oxidation of glucose to carbon dioxide and water may be divided into two phases, (a) glycolysis, the anaerobic process in which glucose is transformed into two molecules of lactic acid, and (b) the aerobic process by which lactic acid or, more correctly, its oxidation product, pyruvic acid, is oxidized aerobically to carbon dioxide and water. In general, when the phrase "burning of carbohydrate" is used, reference is made to the aerobic oxidation of pyruvic acid rather than to the preliminary glycolysis of hexose.

The details of the sequence of reactions by which pyruvic acid is burned to carbon dioxide and water have been worked out by the combined efforts of many investigators, notably Krebs, Martius, Peters, Ochoa, Breusch, Wieland, Wood, Werkman, Weinhouse, Gurin and Evan , and in essence can be summarized in the form of the diagram below representing Krebs' version of the citric acid cycle (1).*



Figure 1

Citric Acid Cycle
(Cf. Reference 2)

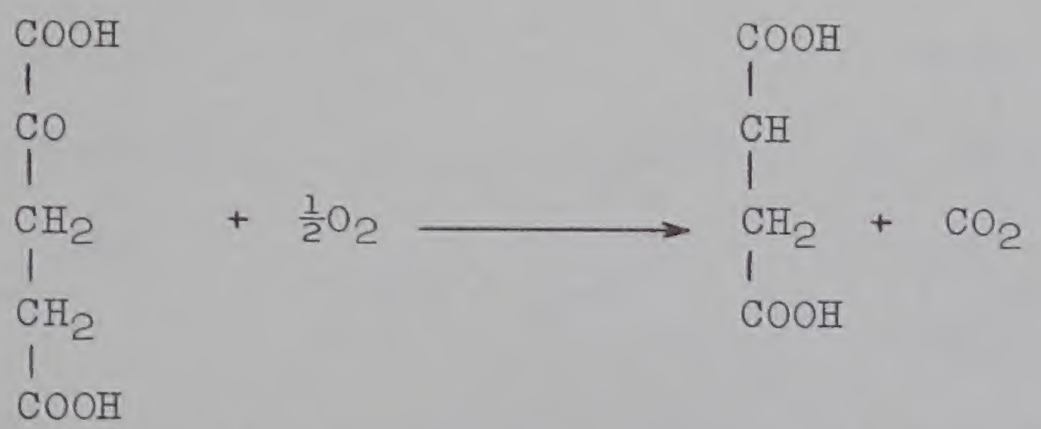
According to this cycle pyruvic acid is not oxidized as such but rather after it has undergone condensation with a molecule of oxaloacetic acid. During one complete turn of the cycle this product of condensation is only partly oxidized to carbon dioxide and water, oxaloacetic acid remaining as the residue. In other words, the equivalent of a molecule of pyruvic acid is oxidized whilst a molecule of oxaloacetic acid is regenerated at each complete turn of the cycle. To start the process going, at least one molecule of oxaloacetic has to be present for condensation with pyruvic acid. Once the reaction is under way, there is constant regeneration of oxaloacetic acid during each turn of the cycle.

The system responsible for catalyzing the entire Krebs' citric cycle has been obtained in cell-free form from liver, kidney, heart muscle and other tissues and it has become possible to study separately each of the many reactions involved in the overall process (2, 3). Before considering the properties of this cell-free system which has been variously named the cyclophorase system, the oxaloacetic oxidase system, and the oxidase system of the insoluble residue, we shall consider the detailed chemistry of each reaction in the cycle insofar as it has been elucidated. For purpose of convenience, the take-off point will be the oxidation of α-ketoglutaric acid but it is immaterial where a beginning is made since the overall reaction is cyclical in nature. It should also be borne in mind that results obtained with enzymes which are not associated with the cyclophorase system may have no bearing on the properties of similar enzymes of the cyclophorase system. The evidence presented in this chapter has been drawn almost exclusively from studies on the cyclophorase system.

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*It does not lie within the province of this chapter to cover the extensive literature dealing with isotope studies on the whole animal or on tissue slices. The emphasis of the present chapter will be on studies with controlled enzyme systems. However, it should be stressed that the isotope studies, particularly those of the Wood and Gurin schools, have provided the solid experimental basis for the citric acid cycle. A most excellent review of the isotope work is provided by H. G. Wood in Physiological Reviews, 26, 198 (1946).

In presence of M/30 malonate (cf. Ochoa (3)), the cyclophorase system catalyzes the oxidation of α-ketoglutarate to succinate according to the following equation:



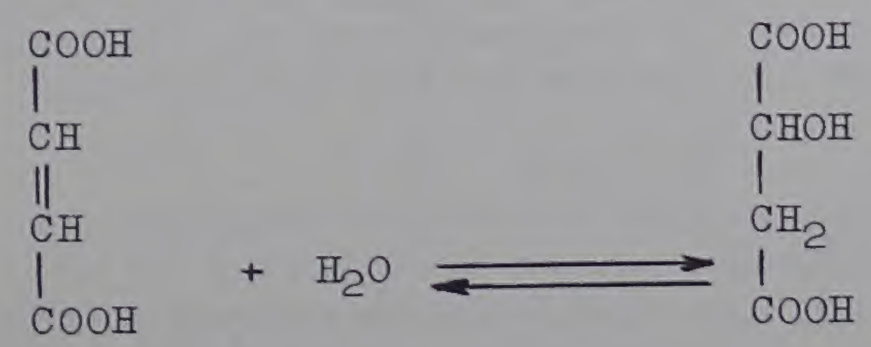
In absence of malonate, a reagent which poisons the succinic dehydrogenase, the oxidation proceeds beyond the stage of succinic acid.

A preparation of the kidney cyclophorase system which has been exposed to a temperature of 38° for about 20 minutes loses the capacity to catalyze all but one oxidation reaction of the cycle, viz., the oxidation of succinic acid to fumaric acid:



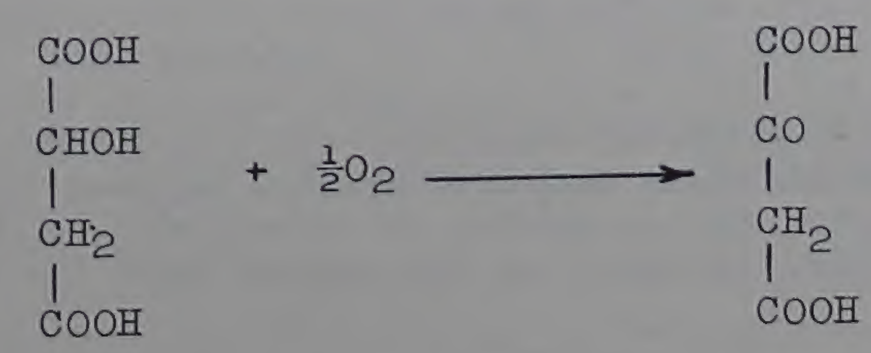
Another device for isolating this reaction involves the use of arsenite as inhibitor (2, 4). At M/30 concentration, arsenite permits the oxidation of succinic to fumaric acid to take place but completely inhibits the subsequent oxidation of fumaric acid, or, more accurately, of its product of hydration, malic acid.

Fumaric acid is brought into equilibrium with L-malic acid by means of an enzyme known as fumarase (5 - 7):



At pH 7 and 38°, the equilibrium ratio of malate to fumarate is about 7.6 (2, 7).

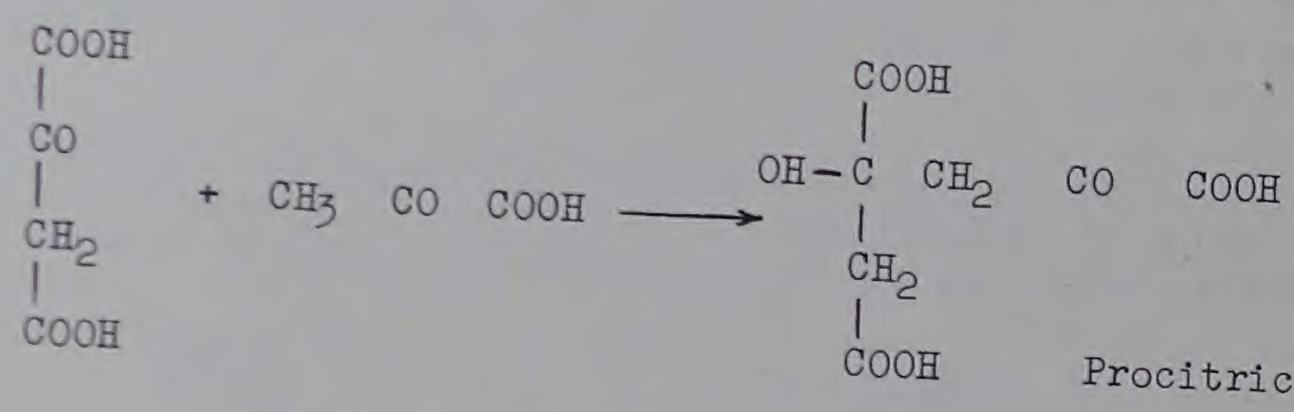
The one-step oxidation of malate to oxaloacetate can be isolated by the expedient of adding 0.1 M HCN, a reagent which combines almost instantaneously and tenaciously with oxaloacetic acid and thereby prevents its further reaction (2, 8).



Oxaloacetic acid can undergo either of two reactions. In presence of the kidney cyclophorase system, it may undergo decarboxylation to pyruvic acid,



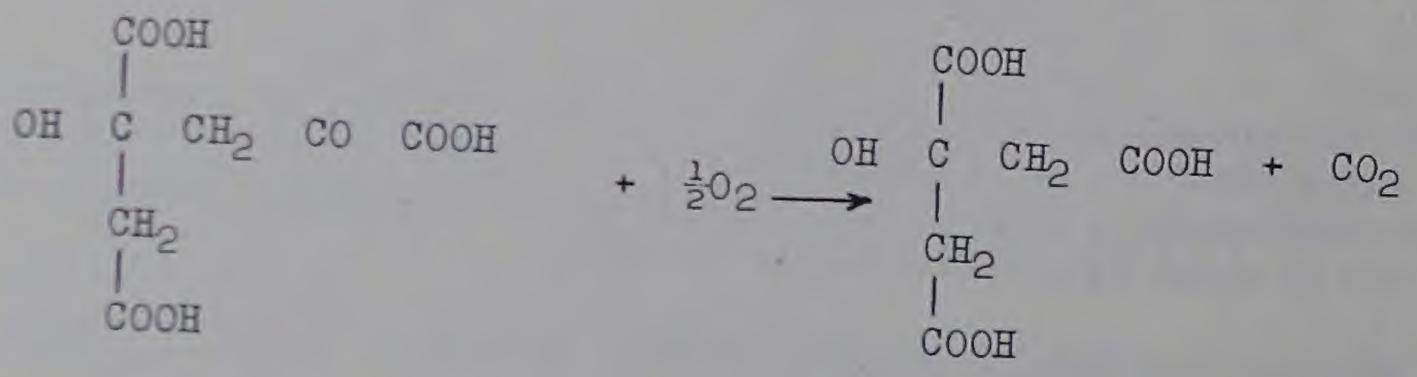
or it may condense with another molecule of pyruvic acid, perhaps in the following fashion:



Procitric acid

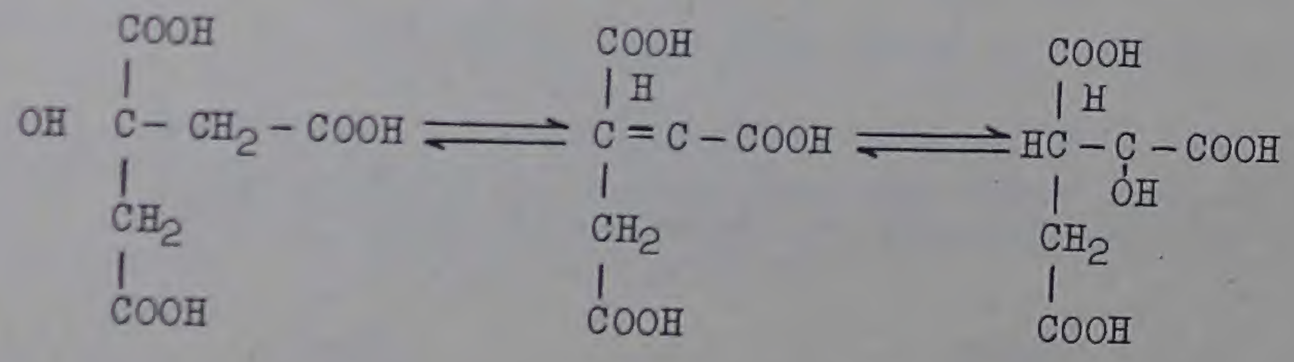
As yet, procitric acid is a hypothetical intermediary. However, the fact that pyruvic and oxaloacetic acids undergo condensation is beyond dispute (9).

Procitric acid is presumed to give rise to citric acid by oxidative decarboxylation:



It must be pointed out that this reaction is still in the realm of paper chemistry. All that is known is that an oxidation is necessary for the conversion of pyruvic and oxaloacetic acids to citric acid but the nature of the intermediary product is still unknown (2, 9, 10).

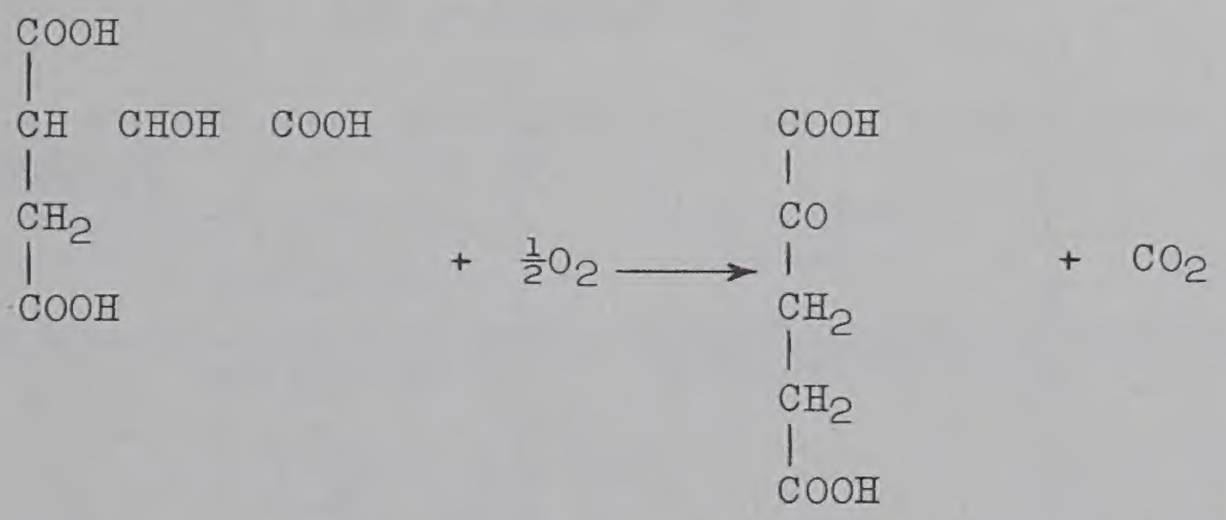
Citric acid is in equilibrium with two other compounds, viz., cis-aconitic acid and l-isocitric acid (11). This equilibrium is established in the presence of the kidney or liver cyclophorase system and can proceed under anaerobic conditions (2):



At pH 7.4 and 38°, the equilibrium ratio of citric to isocitric to cis-aconitic is about 89 to 8 to 3 (1, 12). In view of this equilibrium it becomes difficult to decide which of the three forms is first produced by oxidation of procitric acid. In the present discussion the term citric acid is used loosely in the sense that it embraces all three equilibrium forms.

One of the citric acid equilibrium forms, presumably isocitric acid, can be shown to be oxidatively decarboxylated to α-ketoglutaric acid in presence of the kidney cyclophorase

system. In presence of buffer of pH 9 (2, 4) the subsequent oxidation of α-ketoglutaric acid to succinic acid is essentially stopped:



Having selected α-ketoglutaric acid as our point of departure we have now described one complete turn of the cycle. Since there are five atoms of oxygen taken up during one turn of the cycle whilst eight atoms of oxygen are necessary for the complete oxidation of α-ketoglutaric acid it necessarily follows that more than one turn of the cycle is necessary for complete oxidation of α-ketoglutaric acid. Thus if each of the successive reactions starting with the oxidation of α-ketoglutaric to succinic acid is represented by the numerals I - V, the following infinite series can be constructed:

	ATOMS OF OXYGEN			
	1st turn	2nd turn	3rd turn	4th turn
I	1	$\frac{1}{2}$	$\frac{1}{4}$	1/8
II	1	$\frac{1}{2}$	$\frac{1}{4}$	1/8
III	1	$\frac{1}{2}$	$\frac{1}{4}$	1/8
IV	$\frac{1}{2}$	$\frac{1}{4}$	1/8	1/16
V	$\frac{1}{2}$	$\frac{1}{4}$	1/8	1/16
	4	2	1	0.5
	n	$\frac{n}{2}$	$\frac{n}{4}$	$\frac{n}{8}$

In the practical limit, i.e., after some ten turns of the cycle 8 atoms of oxygen will be taken up for each molecule of α-ketoglutaric acid oxidized and the oxidation to carbon dioxide and water will be complete. It can readily be demonstrated that all other members of the cycle undergo complete oxidation to carbon dioxide and water (cf. Table I).

Before proceeding to other aspects of the citric acid cycle, mention should be made of the reversibility of at least two of the component reactions. Not only can oxaloacetic acid be decarboxylated to pyruvic acid and carbon dioxide, but there is evidence (cf. Fig. 2) that the reverse, viz., carboxylation of pyruvic acid to oxaloacetic acid, can also take place. Evans, Wood, and Werkman (13 - 15) have studied carbon dioxide fixation by pyruvic acid in soluble enzyme systems obtained from liver and bacteria. It remains to be determined whether the reversibly acting oxaloacetic carboxylase of the kidney cyclophorase system is identical with any of the soluble enzymes described by these investigators.

TABLE I

Complete Oxidation of Members of Citric Acid Cycle
(Cf. Reference 2)

Substrate	Substrate added	Oxygen	
		Observed	Theory for complete oxidation
	micromoles	microatoms	microatoms
α-Ketoglutarate	5	38.1	40.0
Malate	5	30.7	30.0
Malate	10	56.8	60.0
Citrate	5	47.1	45.0
Isocitrate	5	45.3	45.0
Succinate	5	34.3	35.0
Fumarate	5	34.0	30.0
Pyruvate	5	23.9	25.0
cis-Aconitate	5	48.4	45.0
Oxalacetate	2.5	12.2	12.5

Substrate	Substrate oxidized	Carbon dioxide	
	micromoles	micromoles	micromoles
α-Ketoglutarate	4.37	21.3	21.9
Isocitrate	3.79	21.7	22.9
Succinate	3.77	15.1	17.2
Fumarate	4.73	16.4	18.9
cis-Aconitate	5.00	27.9	30.0

The complete system in every case contained 1.5 cc. of Residue R_3K, 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M magnesium sulfate, 0.2 cc. of 0.125 M phosphate buffer of pH 7.2, and substrate in the amount indicated. In the main compartment of both the experimental and blank manometer vessels, 1 micromole of succinate was added to prevent the deterioration of the enzyme during the equilibration period. After 5 minutes, the taps were closed and the substrate solution in the side arm was then mixed with the cup contents.

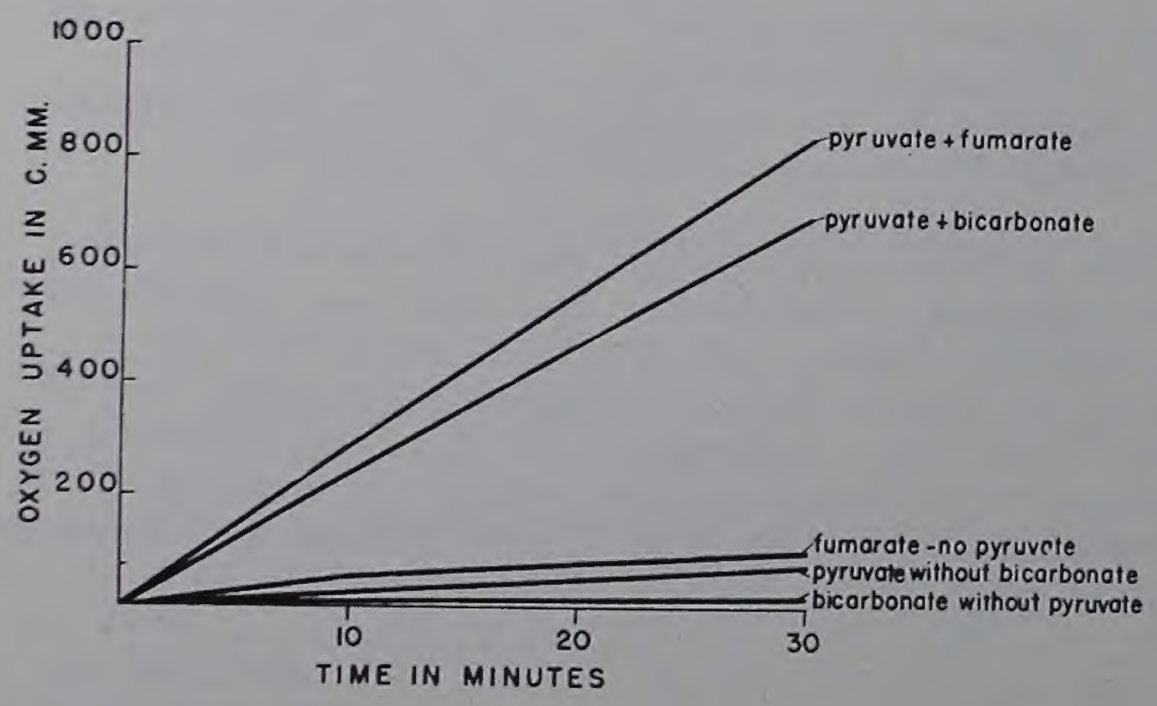
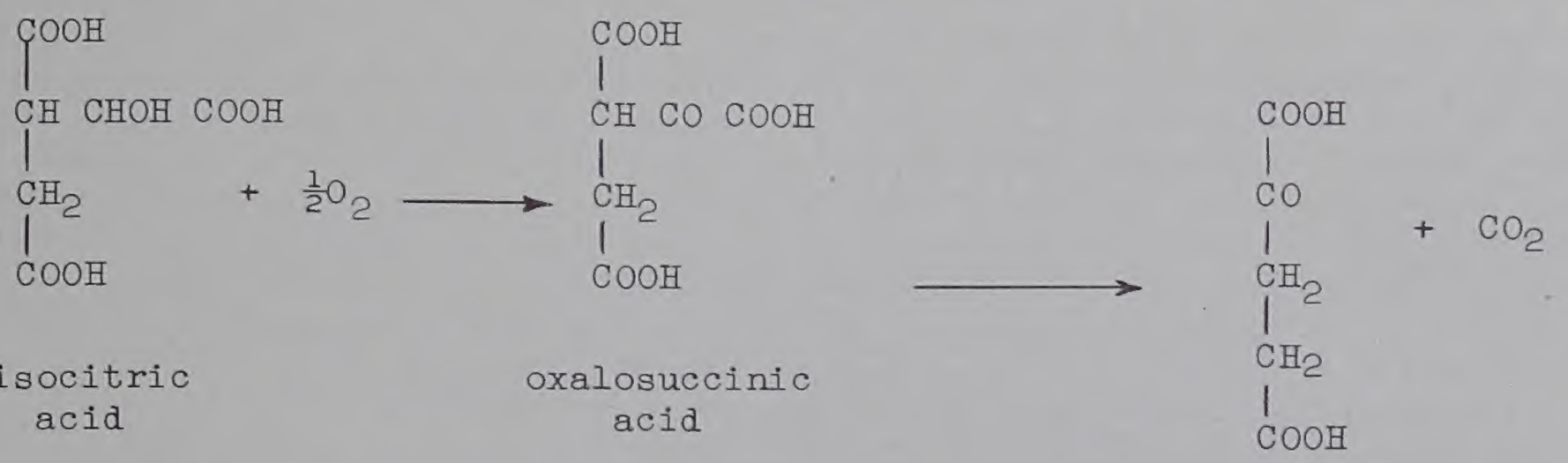


Figure 2
(Cf. Reference 2)

Sparking of pyruvate oxidation with bicarbonate or with fumarate in catalytic concentration. The complete systems contained 1 cc. of Residue R_3K, 0.5 cc. of 0.1 M pyruvate, 0.3 cc. of 0.01 M adenosine triphosphate, 0.2 cc. of 0.02 M $MgSO_4$, 0.1 cc. of 0.125 M phosphate buffer of pH 7.3, and either 0.5 cc. of 9.1 M bicarbonate or 0.03 cc. of 0.1 M fumarate.

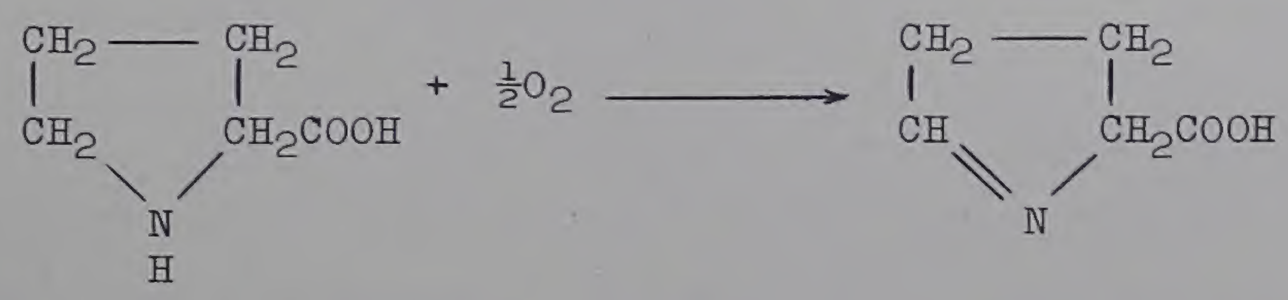
The oxidation of isocitric acid to α-ketoglutaric acid, catalyzed by a soluble enzyme from heart which requires triphosphopyridinenucleotide for activity, has been shown by Ochoa (16) to proceed in two distinct steps:

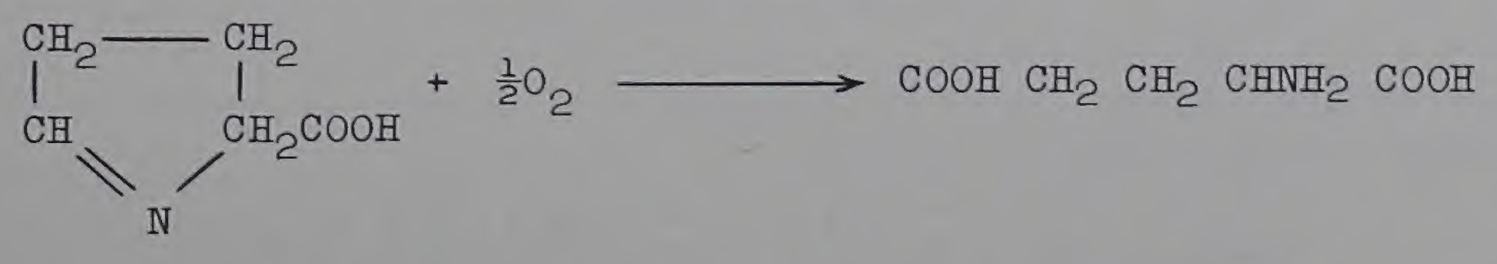


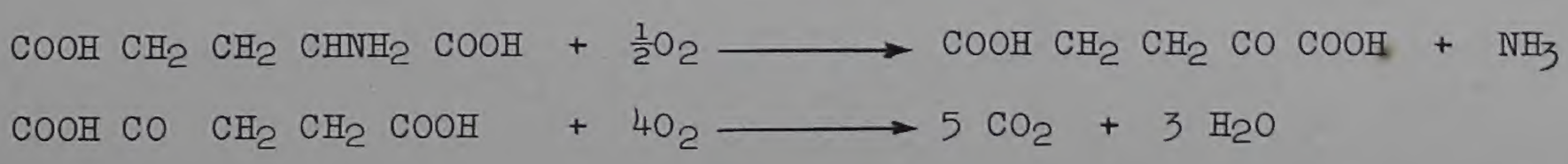
isocitric acid — oxalosuccinic acid — α-ketoglutaric acid

The first reaction is catalyzed by the dehydrogenase system while the second is catalyzed by a carboxylase apparently specific for oxalosuccinic acid. Under appropriate conditions, the reaction can be reversed, i.e., α-ketoglutaric acid can be carboxylated to form oxalosuccinic acid. The equilibrium, however, is almost entirely in favor of decarboxylation. There is no positive evidence that an oxalosuccinic carboxylase is present in the kidney cyclophorase preparation. Oxalosuccinic acid undoubtedly arises as the first product of oxidation of isocitric acid but under the conditions used in studying the cyclophorase system, the β-ketoacid loses carbon dioxide spontaneously. This spontaneous rate is adequate to prevent any appreciable accumulation of oxalosuccinic acid during the oxidation of isocitric acid.

Associated with the cyclophorase system prepared from kidney and liver, there are enzymes which catalyze the oxidation of L-proline to α-ketoglutaric acid, the latter in turn being oxidized to carbon dioxide and water in the cyclophorase system. Taggart and Krakauer (17) have demonstrated that the following series of reactions take place:







Thus a mechanism is provided whereby two amino acids, viz., L-proline and L-glutamic acid can be oxidized to completion eventually by way of the citric acid cycle. L-Hydroxyproline is also oxidized but the rate is rather small compared to that of proline and the oxidation appears to be only partial. The enzymes responsible for these oxidations are invariably found in association with the cyclophorase systems of both liver and kidney. More recently, an enzyme which catalyzes the oxidation of L-alanine has been found in association with the cyclophorase system (18).

The fatty acids represent a third group of substances which can be oxidized to completion in the presence of preparations from liver and kidney containing active cyclophorase systems (19). Not only does fatty acid oxidation have to proceed to completion by way of the citric acid cycle, but in addition, fatty acid oxidation has to be initiated or sparked by the simultaneous operation of the citric acid cycle. The full significance of this initiation process will be considered in a later section. Thus, butyric acid is inert in the presence of a kidney or liver enzyme system unless a catalytic amount of some member of the citric acid cycle is also added simultaneously (19a). At the end of the reaction the oxygen uptake and carbon dioxide formation will be found to correspond to the complete oxidation of both butyrate and the catalyst (usually referred to as sparker). Table II lists the fatty acids which can be oxidized by the liver and kidney systems, and their products of oxidation. All the even numbered fatty acids from C_2 to C_{12} are oxidized to completion. Propionic acid is not oxidized in the kidney preparation. All the odd numbered fatty acids from C_5 to C_{13} are oxidized to propionic acid, carbon dioxide and water. Similarly, isobutyric acid is formed by oxidation of isocaproic acid. Phenyl fatty acids are also oxidized but because of the low rate with which they are acted upon by the fatty acid oxidase system, it has not yet been possible to determine the nature of the end products of oxidation.

TABLE II

Fatty Acids Oxidized by Rabbit Kidney Cyclophorase Preparation
(Cf. Reference 19)

Fatty Acid	Oxidation Products
Acetic	CO_2 and water
Butyric	CO_2 and water
Valeric	Propionic acid, CO_2 and water
n-Caproic	CO_2 and water
Isocaproic	Isobutyric acid, CO_2 and water
Heptylic	Propionic acid, CO_2 and water
Octanoic	CO_2 and water
Pelargonic	Propionic acid, CO_2 and water
Capric	CO_2 and water
Undecylic	Propionic acid, CO_2 and water
Lauric	CO_2 and water
Tridecylic	Propionic acid, CO_2 and water
$\Delta^{10\text{-}11}$-undecylenic	Acrylic acid, CO_2 and water

Before proceeding to the detailed intermediary chemistry of fatty acid oxidation, a few remarks are in order about the historical development of this field. The β-oxidation scheme of Knoop (20) which was worked out in whole animal feeding experiments, has been the foundation for the present conception of how fatty acids are oxidized in the animal body. Knoop showed that phenyl substituted fatty acids are degraded in the animal body in the following type of sequence:

$$\begin{array}{l} RCH_2 - CH_2 - COOH \\ \quad\quad\downarrow \\ RCH = CH - COOH \quad \rightleftharpoons \quad R\ CHOH\ CH_2\ COOH \\ \quad\quad\downarrow \\ R - CO - CH_2 - COOH \\ \quad\quad\downarrow \\ R\ COOH + CH_2\ COOH \end{array}$$

β-Oxidation was considered by Knoop as a device for chopping down a fatty acid in cuts, so to speak, of a two-carbon acetic acid unit. He postulated that acetoacetic acid arose exclusively from butyric acid by β-oxidation. It was not until very recently that fatty acid

oxidation was demonstrated in cell-free systems by Leloir and Munoz (21) in a now classical investigation. Others who have made important contributions to our knowledge of the enzymes involved and the intermediary reactions are Lehninger (22 - 29), Potter (30), Quastel (31), Cohen (31a), Weinhouse (32), and Gurin (33).

If the initial phases of fatty acid oxidation, as catalyzed by the liver and kidney cyclophorase systems, proceeded according to the classical Knoop scheme, we should expect not only butyric acid, for example, to be oxidized to completion, but also crotonic acid, β-hydroxybutyric acid and acetoacetic acid. Under appropriate conditions, all these postulated intermediates of fatty acid oxidation are indeed oxidized to completion at rates comparable to that of butyric acid. In contrast, α-hydroxybutyric acid, γ-hydroxybutyric acid, α, β-dihydroxybutyric acid and α-ketobutyric acids are not acted upon by the kidney cyclophorase system. It is of interest to note that isocrotonic acid, which is the cis isomer of trans-crotonic acid, and vinylacetic, the Δ β, γ-unsaturated butyric acid are both oxidized as readily as trans-crotonic acid. Perhaps the most direct evidence that the oxidation of butyric acid in the cyclophorase system proceeds according to the Knoop scheme is the demonstration by Knox and Weinhouse (34) that when butyric acid labeled in the carboxyl group with C^{13} is oxidized in presence of unlabeled acetoacetic acid, there is found at the end of the experiment the label exclusively in the carboxyl group of acetoacetic acid. In other words, butyric is oxidized by way of acetoacetic acid. A similar experiment was carried out in a system oxidizing labelled octanoic acid in presence of an excess of acetic acid. At the end of the run acetic acid was isolated and found to contain isotope, thus demonstrating the essential step of β-oxidation.

The derivatives which would arise by β-oxidation of butyric, valeric, isocaproic, hexanoic, and octanoic acids have been tested and they are all oxidized to completion and at rates comparable to those of the parent fatty acids (cf. Table III). There would appear to be little doubt that in the cyclophorase system, fatty acids are oxidized first by way either of the α, β-unsaturated acids or the β-hydroxy acid and then by way of the β-keto acid.

TABLE III

Oxidation to Completion of β-Hydroxy, β-Keto and α-β Unsaturated Fatty Acids by the Kidney System

(cf. Reference 19)

Fatty Acid Derivative	μ mols added	μ mols utilized	μ atoms oxygen absorbed (blank subtracted)	Theory for oxidation to CO_2 and H_2O	Theory for oxidation to non-oxidizable residue and CO_2 and H_2O
d,l-β-Hydroxybutyric	3		26.2	27	
d,l-β-Hydroxyvaleric	2.5		11.8		12.5[a]
d,l-β-Hydroxyisocaproic	2		10.0		10.0[b]
d,l-β-Hydroxycaproic	1.5		21.0	22.5	
d,l-β-Hydroxyoctanoic	1		21.4	21.0	
β-Ketovaleric	2		8.1		8.0[a]
β-Ketoisocaproic	10	3.30	13.8		13.2[b]
β-Ketooctanoic	7.5	3.84	33.8	38.4	
Isocrotonic	6		62.3	54	
$\Delta^{2,3}$-pentenoic	5		23.8		25[a]
4-Methyl-$\Delta^{2,3}$-pentenoic	2.5		14		12.5[b]
Trans $\Delta^{2,3}$-hexenoic	4		62.3	60	
Cis $\Delta^{2,3}$-hexenoic	3		41.2	45	
Sorbic	5		70	70	

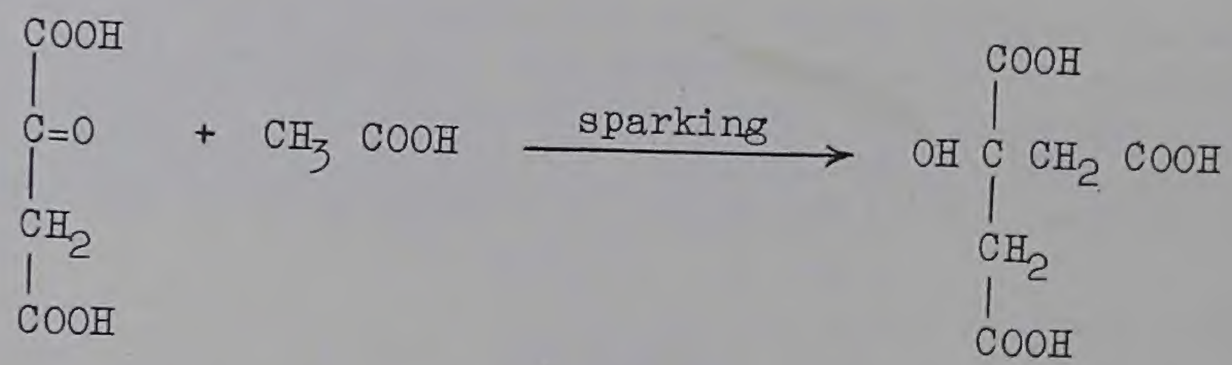
[a] residue is propionic acid
[b] residue is isobutyric acid

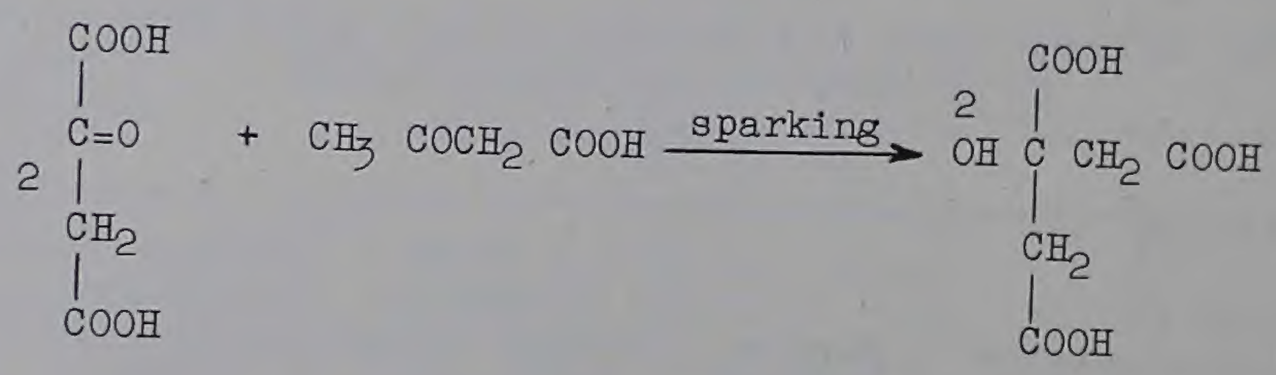
The complete system contained M/100 adenylic acid, 5 μ mols of α-ketoglutaric acid, M/150 phosphate buffer of pH 7.3, M/150 magnesium sulfate and 1.5 cc. of enzyme at the R_3 residue stage. Oxygen in gas space; 38°.

The evidence is also straightforward that fatty acids are degraded by stepwise removal of an acetic unit. Thus, valeric acid is oxidized to propionic acid, and isocaproic acid is oxidized to isobutyric acid (cf. Atchley (35)). In both these cases, one acetic unit is chopped off and the oxygen uptake corresponds to the complete oxidation of acetic acid to carbon dioxide and water.

The details of fatty acid oxidation can now be systematized in terms of the following generalizations: (1) Fatty acids are degraded by β-oxidation with the intermediary formation of a C_2 acetic acid unit. (2) When an even numbered fatty acid is oxidized in the kidney system the process of beta oxidation leads exclusively to the formation of acetic acid units as end products. (3) When an even numbered fatty acid is oxidized in the liver system, beta oxidation leads to the chopping off of acetic units until the stage of acetoacetic acid is reached. Both acetoacetic and acetic acids are the end products of β-oxidation in the liver system. (4) When an odd numbered fatty acid or a fatty acid with a non-oxidizable residue such as acrylic acid is oxidized in the kidney system, the products of β-oxidation are acetic acid and the non-oxidizable residue.

The mechanism of complete oxidation of fatty acids now involves the details by which acetic acid and acetoacetic acids are oxidized in the cyclophorase system. Both acetic and acetoacetic acids have been shown to condense with oxaloacetic acid to form some tricarboxylic acid, presumably citric acid, which in turn, is oxidized to completion in the cyclophorase system. The details of these two condensations have yet to be fully worked out but provisionally they may be represented as follows:





The pioneer investigations on these condensations were carried out by Breusch (9, 36), Wieland (37), Lynen (38), and Hunter and Leloir (39). There is one remarkable feature about both these condensations. They do not proceed unless simultaneously some intermediate in the citric acid cycle is undergoing oxidation. Here again we are dealing with a sparking or priming phenomenon, consideration of which we shall have to defer for the moment. It might be mentioned that barium ions in high concentration have been used to prevent the condensation from proceeding beyond the citric acid stage (37). Otherwise it would be impossible to demonstrate citric acid as one of the products of the condensation reactions.

Oxaloacetic acid is now known to condense with three different partners to form tricarboxylic acid. The condensation with pyruvic acid lies within the province of the citric acid cycle whereas the condensations with acetic and acetoacetic acids provide the links between fatty acid oxidation and the citric acid cycle.

By controlling conditions, the oxidation of fatty acids in the liver cyclophorase system may either be allowed to proceed to completion or it may be arrested at the stage of acetoacetic acid (19). The mechanism by which acetoacetic acid arises in the liver cyclophorase system can be shown to involve a) formation of butyric acid by degradation of higher even numbered fatty acids and b) β-oxidation of butyric acid to acetoacetic. Only

TABLE IV

Acetoacetic Acid Formation During Fatty Acid Oxidation in Liver
(cf. Reference 19)

Each of the fatty acids was tested in the presence of 1.5 cc. of liver enzyme at the R_1 residue stage, 0.6 cc. of 1 per cent heart extract and 0.3 cc. of 0.01 M α-ketoglutarate. Final volume, 3 cc. oxygen in gas space, 38°. The value for acetoacetic acid found at the end of the experiment in the blank was usually about 1 μ mole or less.

Experiment series	Fatty Acid	μ moles added	μ atoms of oxygen absorbed (corrected for blank)	μ moles of acetoacetic acid formed (corrected for blank)
1	Caproic	40	163	14.8
	β-Hydroxycaproic	40	124	13.1
	trans $\Delta^{1,2}$-Hexenoic	40	119	15.1
	Sorbic	40	84	12.5
2	Butyric	50	40	5.4
	Crotonic	50	21	6.6
	Isocrotonic	50	34	6.6
	Vinyl acetic	50	32	7.2
	β-Hydroxybutyric	50	22	13.6
3	Caproic	40	154	15.0
	Octanoic	20	132	12.8
	Valeric	60	79	2.1
	Heptylic	30	114	3.4
	Isocaproic	60	93	1.4
4	Caproic	40	163	14.8
	Valeric	60	99	0.4
	$\Delta^{1,2}$-Pentenoic	60	75	0.3
	Isocaproic	60	80	1.1
	4-Methyl $\Delta^{1,2}$-Pentenoic	60	105	0.4

the even numbered fatty acids containing four or more carbon atoms can give rise to appreciable amounts of acetoacetic acid (cf. Table IV). The odd-numbered or branched chain fatty acids yield little or no acetoacetic acid during their oxidation although they are oxidized as readily as the even-numbered fatty acids.

The whole animal experiments of McKay (40) and the isotope experiments of Weinhouse (32) have established that acetoacetic acid can also arise as a condensation product of acetic. Lehninger (24) has described a system in rat liver which converts octanoic acid quantitatively to acetoacetic acid according to the equation:

$$C_3(CH_2)_6\ COOH + 6O \longrightarrow 2CH_3CO\ CH_2\ COOH$$

Here the acetoacetic acid formed must arise from two sources: (1) from butyrate by β-oxidation and (2) from acetic acid by condensation.

The special feature of Lehninger's system is that it must include, in addition to the enzymes responsible for implementing β-oxidation, the enzyme which condenses acetic acid to acetoacetic acid. It is to be noted that acetic acid as such is not the primary product of fatty acid degradation. Therefore the condensation must be thought of in terms of an

activated acetic acid unit. It has yet to be determined whether this enzyme which forms acetoacetic acid by condensation of acetic acid is present in kidney and other tissues.

Lehninger (24) has also described in rat liver extracts, an enzyme system which oxidatively converts pyruvic acid into acetoacetic acid according to the following equation:

$$2\ CH_3\ CO\ COOH + O_2 \longrightarrow CH_3\ CO\ CH_2\ COOH + CO_2 + H_2O$$

It appears likely that the overall reaction consists of two separate reactions:

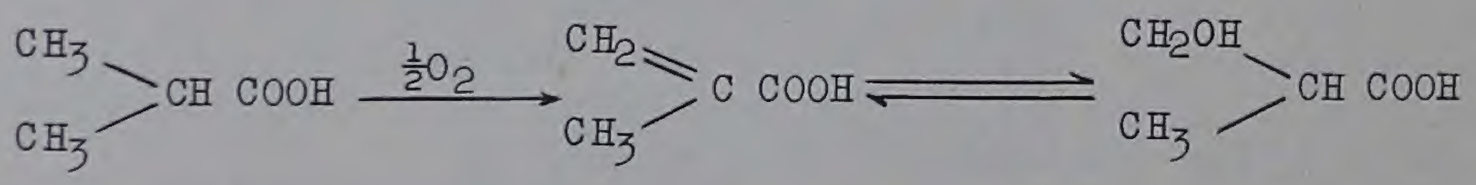
$$2\ CH_3\ CO\ COOH + O_2 \longrightarrow 2\ CH_3\ COOH^* + CO_2$$

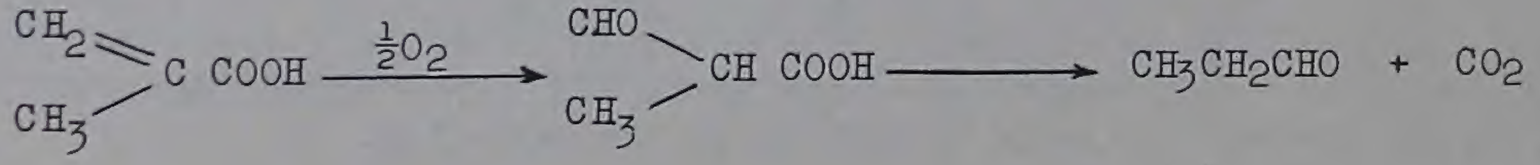
$$2\ CH_3\ COOH^* \longrightarrow CH_3CO\ CH_2COOH + H_2O$$

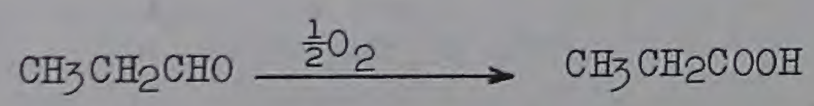
The asterisk over the acetic acid indicates that it is not ordinary acetic acid but a specially reactive form. The condensing enzyme is without effect on acetic acid as such. However, Knox and Weinhouse (34) have shown that when the oxidation of pyruvic acid to acetoacetic acid is conducted in the presence of acetic acid labelled with C^{13} in the carboxyl group, the label appears in the acetoacetic acid equally distributed between the carboxyl group and the β-carbon atom. This experiment reveals a most significant fact, viz., that activation of acetic acid is an event which comes subsequent to the oxidation. If activation of acetic acid were inextricably linked up with oxidation of pyruvic acid, it would be impossible to incorporate into acetoacetic acid the label contained in acetic acid which did not arise from pyruvic acid by oxidation. A reasonable interpretation is that oxidation of pyruvic acid leads to the formation of some activating complex which can activate acetic acid regardless of whether it arises from pyruvic acid by oxidation or is present as such in the medium. It is possible that the same condensing enzyme catalyzes the formation of acetoacetic acid from activated acetic acid whether the activation of acetic acid is brought about during fatty acid oxidation or during oxidation of pyruvic acid.

Liver contains an enzyme system which catalyzes the α-oxidation of propionic and phenylpropionic acids leading in both cases presumably to the formation of the corresponding hydroxy acids (41). Since lactic acid can be converted by another oxidase to pyruvic acid, it becomes possible to accomplish the complete oxidation of propionic acid in the liver cyclophorase system. The propionic acid oxidizing enzyme is not found in the kidney cyclophorase preparation.

Both liver and kidney cyclophorase systems contain a group of enzymes which can convert isobutyric acid to propionic acid according to the following mechanism (35):







The oxidation of isobutyric acid is not rapid and it is possible to demonstrate both propionic and isobutyric acid as intermediates in the oxidation of isocaproic acid.

The method of preparing the cyclophorase system from rabbit kidney or liver consists essentially in mincing the tissue in 0.9% potassium chloride at 0° in a blendor and adding alkali in an amount just sufficient to neutralize the acid formed when the cells are rup-

tured. The easily sedimentable fraction of the resulting homogenate contains the cyclophorase system, and this fraction is purified by repeated suspension in potassium chloride solution followed by centrifugation. The first residue obtained by centrifuging the homogenate is called R_1, the second R_2, etc. In general the "enzyme" is brought to the third residue stage. The QO_2 of the rabbit kidney cyclophorase system catalyzing the citric acid cycle is about 30.

The preparation of the cyclophorase system from rabbit kidney is active over a period of 12 hours when stored at 0°. The system is destroyed by freezing, drying, exposure to high concentration of salt (greater than 0.1 M), to solvents, to deionized water, to acidity below that of pH 4.0 or to alkalinity above that of pH 10.

The enzyme suspension is a highly viscous, faintly yellow-colored gel. Procedures or conditions which lead to inactivation of the cyclophorase system in many cases lead to visible alteration in the appearance of the gel from its characteristic smooth consistency to one that resembles curdled milk.

There is evidence (42) that it consists largely, if not exclusively, of nucleoprotein* of very high molecular weight, but whether these molecules of nucleoprotein contain all the enzymes responsible for implementing the oxidation of pyruvic acid and other metabolites to carbon dioxide and water remains a problem. The amazing constancy of the activity ratios of these many enzymes in successive preparations of the cyclophorase system and the invariant association of these many enzymes would argue that they are all tied up with the same structural units so that no one of them can be separated out from the rest. It could hardly be mere coincidence that this extraordinary variety of enzymes, all of which have to work either in sequence or in parallel, should always be found together in the same suspension of particles.

When the cyclophorase system of kidney or liver is freshly prepared, nothing has to be added to the system except molecular oxygen in order to obtain the complete oxidation of any member of the citric acid cycle, of proline and glutamic acid , and of fatty acids. In other words, all the enzymes and coenzymes are both present and intact. With aging of the preparation for one or two hours before testing, the rate of oxidation is found to fall off rapidly with time unless magnesium ions and adenylic acid (or adenosinetriphosphate) are added (cf. Fig. 3). There appears to be an autolytic process which either splits off

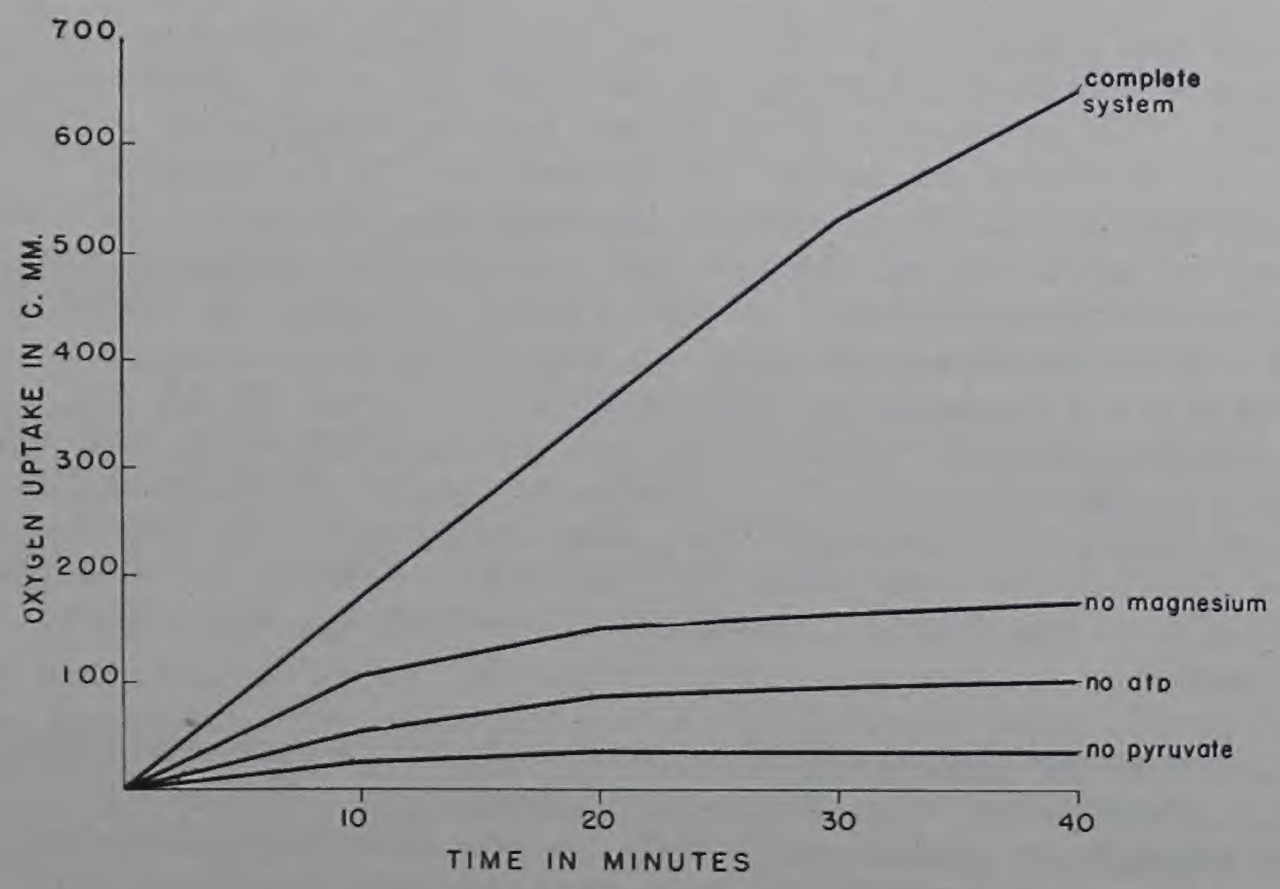


Figure 3
(Cf. Reference 2)

Components required for the oxidation of pyruvate by an aged enzyme preparation. The complete system contained 1 cc. of a 4 hour-old Residue R_3K, 0.5 cc. of 0.1 M pyruvate, 0.2 cc. of 0.02 M magnesium sulfate, 0.3 cc. 0.01 M adenosine triphosphate (atp), 0.5 cc. of 0.1 M sodium bicarbonate, 0.2 cc. of 0.125 M phosphate buffer of pH 7.3, alkali in the center well (0.2 cc.), and water to make up to a final volume of 3 cc. Oxygen in the gas space.

*The cyclophorase system as prepared by the standard procedure contains approximately equal amounts of desoxy and ribose nucleic acid nucleoproteins. According to Lehninger (43) and Schneider (42) the catalytic activity is associated exclusively with the ribose-nucleic acid nucleoproteins which appear to be derived from the mitochondrial fraction of the cytoplasm.

magnesium and adenylic acid from the cyclophorase protein or leads to some change which can be corrected only by addition of magnesium and adenylic acid. That the process is autolytic in nature is confirmed by the fact that in presence of sodium fluoride the requirement for adenylic acid is greatly reduced (2). All but one oxidative step in the citric acid cycle require the addition of adenylic acid. There appears to be no adenylic requirement for the step involving oxidation of succinate to fumarate. Several other enzymes associated with the cyclophorase system show this requirement for adenylic acid, notably the glutamic dehydrogenase, β-hydroxybutyric dehydrogenase and the oxidase which catalyzes the oxidation of pyruvic acid to acetoacetic acid (44).

The oxidations catalyzed by the cyclophorase and associated systems are usually carried out in presence of phosphate ions, but, unless special conditions are imposed which will be considered later, these reactions proceed just as well in the complete absence of added phosphate.

When the kidney enzyme system at the third residue stage (R_3K) is allowed to oxidize α-ketoglutaric acid or any other member of the citric acid cycle in presence of the usual components some disappearance of inorganic phosphate is observed. There is no disappearance in absence of added substrate and less disappearance when components of the system are omitted. Fluoride in 0.01 M concentration significantly augments the amount of inorganic phosphate which disappears. The amount of phosphate which disappears is extremely variable from one enzyme preparation to another. In addition, the time curve shows that within the first few minutes of the experiment the amount of inorganic phosphate which disappears is about 50 per cent of that observed at the end of one hour. It thus appears that there are two competing processes operating simultaneously, one involving the disappearance of inorganic phosphate and the other the regeneration of inorganic phosphate.

The disappearance of inorganic phosphate is found to be accompanied by the formation of a labile phosphoric ester which is completely hydrolyzed following 7 minute exposure to 1 N acid at 100°. The phosphoric ester has been isolated from the reaction mixture and identified as inorganic pyrophosphoric acid (Cross et al. (44) and Cori and Ochoa (45)). With the identification of inorganic pyrophosphoric ester as the product into which inorganic phosphate is transformed, it became clear that the competing process was in fact the hydrolysis of the ester by the pyrophosphatases present both in the liver and kidney enzyme systems. Fluoride suppressed the activity of the pyrophosphatases but did not abolish it altogether under the conditions of the experiment.

An actively functioning cyclophorase system at the third residue stage produces as much inorganic pyrophosphate from phosphate whether glucose is present or not. However, with an enzyme at the first residue stage, the presence of glucose has a pronounced effect; little inorganic pyrophosphate accumulates whereas an ester is formed which is alkali labile and which generally has the characteristics of a hexose phosphate. Apparently some enzyme essential for the phosphorylation of glucose is washed out during the preparation of the cyclophorase system. Indeed, when the supernatant of the first residue is added to an enzyme at the third residue stage it restores the capacity to phosphorylate glucose. Experiment has shown that yeast hexokinase as prepared by the method of Colowick *et al.* (46) and muscle hexokinase as prepared by the method of Cori *et al.* (47) reproduce the effect of the supernatant enzyme completely. Hexokinases as a class transfer phosphate from adenosine polyphosphate to some hexose or hexosemonophosphate. The inference is therefore clear that adenylic acid is acting as a shuttle between the cyclophorase system on the one hand which acts as the donor of phosphate and the hexokinaseglucose system on the other hand which acts as the acceptor of phosphate.

Magnesium ions, adenylic acid (or adenosine triphosphate) and substrate are essential for the over-all conversion of inorganic phosphate into hexosephosphate (*cf.* Table V). Fluoride again is essential for maximum phosphorylation although in the concentration used it lowers the rate of oxidation. Since the requirements of two systems are involved in the over-all phosphorylation process, it does not follow that the components needed for phosphorylation are identical with those needed for the operation of the cyclophorase system.

Kalckar *et al.* (48) have observed in homogenates of rabbit kidney that adenosine and glycerol could serve as phosphate acceptors during oxidation of cycle substrates. Similar-

TABLE V

Components of System for Phosphorylation of Fructose in Kidney Cyclophorase System (cf. Reference 44)

System	Oxygen uptake in μ atoms	Phosphate disappearance in μ mols	$\triangle P / \triangle O$
Complete	13.4	35.6	2.66
No adenylic	5.5	5.7	1.03
No magnesium ions	9.7	10.3	1.06
No fluoride ions	25.1	34.5	1.37
No fructose	13.6	3.7	0.27
No hexokinase	14.7	10.0	0.68

The complete system contained 1 cc. of enzyme at the R_3K residue stage, 0.02 cc. of yeast hexokinase, M/1000 adenylic acid, M/150 magnesium chloride, M/60 phosphate buffer of pH 7.3, M/30 fructose, M/90 sodium fluoride and M/100 α-ketoglutarate. Final volume, 3 cc.

ly, Potter et al. (49) have used creatine as a phosphate acceptor for rat liver homogenates acting upon oxaloacetate. These reactions require special enzymes which are not contained either in the cyclophorase preparations at the stage of the third residue or in the solutions of purified yeast hexokinase.

Considerable theoretical significance attaches to the determination of which oxidative steps involve esterification of inorganic phosphate and of the number of phosphate molecules which are esterified per atom of oxygen absorbed. There is an extensive literature on this subject, the most notable contributions being those of Belitzer (50), Kalckar (51), Colowick (52), Potter (49), and Ochoa (3). These investigators have established the linkage of phosphate esterification with the aerobic oxidation of succinic acid, glutamic acid, oxaloacetic acid and α-ketoglutaric acid. While there is general agreement that more than 1 molecule of inorganic phosphate was esterified per atom of oxygen, the conditions under which these experiments were carried out make it difficult to evaluate the limiting P/O ratio. Ochoa (3) in his study of the one step oxidation of α-ketoglutarate to succinate attained a ratio of 1.8.

It would appear from Table VI that more than two molecules of phosphate are taken up for each atom of oxygen absorbed in every step of the citric acid cycle except that of succinic to fumaric, which has a P/O ratio of less than two. The ratios for the oxidation of glutamate and proline are also above two. Since only whole numbers of phosphate molecules can be esterified per atom of oxygen, it follows that in those cases where the experimentally determined ratio exceeds two that the actual ratio is three and is not attained owing to back reactions as yet unspecified. The observed P/O ratio for the oxidation of β-hydroxybutyrate to acetoacetate appears to be less than two.

On the basis of the P/O ratios for each of the five steps involved in the oxidation of pyruvic acid to carbon dioxide and water, a total of 14 molecules of phosphate should be taken up for each molecule of pyruvic acid oxidized to completion. Lipmann (53) has calculated the maximum number of phosphate molecules (cf. Table VII) which could be esterified during the oxidation of pyruvate. Three assumptions are made in this calculation: (1) that each of the five oxidative steps is completely reversible, (2) that the difference between the oxidation-reduction level at each step and the oxidation-reduction level of the oxygen electrode can serve as the basis of calculating the $\triangle F$ for each step, and finally (3) that 12,000 k cal/mole is the most probable value for the $\triangle F$ value of a pyrophosphate linkage. The calculated value turns out to be 23 moles of phosphate esterified per molecule of pyruvic acid. In his calculation Lipmann has approximated only very grossly the oxidation-reduction potential levels for the α-ketoglutaric to succinic step and for

TABLE VI

Ratios of Phosphate Esterified to Oxygen Consumed with Different Substrates (cf. Reference 44)

Substrate	Method of Determination	Range	Mean Ratio P/O	Number of Determinations	Standard Deviation
α-Ketoglutarate	Extrapolation	1.51-3.43	2.43	62	±0.425
	Interval	2.15-2.46	2.26	5	
Succinate	Extrapolation	0.88-1.55	1.25	26	±0.179
	Interval	1.14-1.40	1.28	3	
Malate	Extrapolation	2.15-2.35	2.25	2	
	Interval	2.16-2.66	2.42	4	
Oxalacetate	Interval	1.94-2.20	2.06	3	
Pyruvate	Extrapolation	2.35-2.59	2.47	2	
Citrate	Extrapolation	0.65-3.26	2.15	22	±0.648
	Interval	1.92-2.99	2.30	3	
Proline	Extrapolation	1.41-2.91	2.07	20	±0.392
	Interval	1.69-2.26	1.97	3	
Glutamate	Extrapolation	1.74-2.88	2.18	19	±0.214
	Interval	2.14-2.46	2.30	2	
β-Hydroxybutyrate	Extrapolation		1.48	1	
	Interval	1.53-1.73	1.64	3	

TABLE VII

Moles of Phosphate Esterified at Each Step in the Citric Acid Cycle (cf. References (53) and (53a)

	Moles Phosphate per Atom Oxygen
α-Ketoglutaric $\xrightarrow{\frac{1}{2}O_2}$ succinic	3
Succinic $\xrightarrow{\frac{1}{2}O_2}$ fumaric	2
Malic $\xrightarrow{\frac{1}{2}O_2}$ oxaloacetic	3
Oxaloacetic + pyruvic $\xrightarrow{\frac{1}{2}O_2}$ citric	3
Citric $\xrightarrow{\frac{1}{2}O_2}$ α-ketoglutaric	3
	14 moles phosphate taken up per mole of pyruvic acid oxidized (5 atoms oxygen)

TABLE VII (Continued)

Theoretical Phosphate/Oxygen Ratio for Oxidation of Pyruvic Acid*
(cf. References (53) and (53a)

	E_h (Volts)	E_h Oxygen Electrode Minus E_h System (Volts)
α-Ketoglutaric ⟶ succinic	-0.78	1.58
Succinic ⟶ fumaric	+0.00	0.80
Malic ⟶ oxaloacetic	-0.18	0.98
Oxaloacetic + pyruvic ⟶ citric	-0.78	1.58
Isocitric ⟶ α-ketoglutaric	-0.30	1.10
		6.04

$\triangle = nEF = 2 \times 6.04 \times 23{,}058$
$= 278{,}000$ calories

$\frac{278{,}000}{12{,}000}$ = 23 moles of pyrophosphate P per mole of pyruvic acid oxidized

$$\text{Efficiency} = \frac{14}{23} \times 100 = 61\%$$

*The data have been taken from the article of Lipmann (53). The values for α-ketoglutaric and oxaloacetic + pyruvic are only gross approximations which may be in error by several hundred millivolts.

the procitric to citric step. These values may be too high by several hundred millivolts which would have the effect of making the calculated phosphate number less than 23. However, by assuming a slightly lower value for the $\triangle F$ of a pyrophosphate linkage, the phosphate number would be proportionately increased beyond 23. Very roughly it may be said that the over-all efficiency of the cyclophorase system when catalyzing the complete oxidation of pyruvic acid is 14/23 x 100 or slightly more than 60 per cent. This is indeed a remarkably high efficiency for processes involving molecular oxygen and would suggest that the coenzyme which is last in the series between the substrate and oxygen must operate at the oxidation level of the cytochromes or even higher in order to allow of such a high efficiency of conversion.

Thus far, experiment has shown that with one exception every oxidation known to take place in the cyclophorase system either of liver or kidney is accompanied by phosphorylation. The one apparent exception concerns oxidation in the fatty acid series which is complicated by the necessity for co-oxidation of some member of the citric acid cycle and also by the sensitivity of fatty acid oxidation to the presence of fluoride. It thus still remains to be shown by direct means that the oxidation of the activated fatty acid to the corresponding unsaturated acid or the oxidation of the activated unsaturated acid to the keto acid is accompanied by phosphate esterification. Lehninger (54) has carried out experiments with radioactive P which suggest that all the processes of β-oxidation are accompanied by phosphate esterification.

A P/O ratio of 3 may signify that in the reaction between substrate and oxygen at least three intermediary coenzymes intervene successively, and that a molecule of phosphate is esterified when each of the three coenzymes undergoes reduction or oxidation. In the light of the formation of inorganic pyrophosphate in absence of a phosphate acceptor system, it may be assumed that coincident with reduction or oxidation, inorganic phosphate is taken up with formation of a coenzyme pyrophosphate which will either split off inorganic

pyrophosphate in absence of an acceptor system or will transfer one phosphate group to some acceptor like adenylic acid.

Oxygen may be replaced by ferricyanide as oxidant for any of the reactions catalyzed by the cyclophorase system. The ratio, moles of phosphate esterified per 2 moles of ferricyanide reduced, never exceeds and only approaches the value of 1 regardless of which substrate is used. If ferricyanide reoxidizes the reduced form of the first coenzyme in the reaction sequence and thus prevents the second and third coenzymes from intervening, it would be anticipated that the limiting ratio would be exactly one.

The oxidation of various substrates like α-ketoglutaric acid can also be accomplished by linking them with the reduction of other substrates. Thus, α-ketoglutarate can be oxidized to succinate at the expense of the reduction of acetoacetate to α-hydroxybutyrate. The experimentally determined values for the ratio, moles phosphate esterified per mole α-ketoglutarate oxidized rarely exceeds a value of about 0.6.

The enzyme which catalyzes the oxidation of succinate to fumarate is the hardiest of all the oxidation enzymes in the cyclophorase complex. Various treatments like exposure of the complex to 38° in absence of added substrate completely inactivate all but the succinic oxidizing enzyme. This hardy quality of the succinic enzyme has helped to uncover new facets in the linkage between oxidation and phosphorylation. Using the P/O ratio as a measure of the linkage phenomenon, it is observed that with progressively longer exposure of the cyclophorase complex to a temperature of 38° in absence of substrate, the ratio becomes progressively smaller reaching 0 as the limit. Apparently the ability to oxidize succinic acid does not suffer in consequence of this heat treatment but the ability to take up inorganic phosphate and eventually transfer the phosphate to glucose via adenosine triphosphate is severely impaired.

The classical investigations of Hotchkiss (56) have implicated gramicidin as well as dinitrophenol as reagents for interrupting the normal link between oxidation and phosphorylation in aerobic systems. In some unpublished experiments quoted in his review article (56), Hotchkiss mentions that he found that gramicidin and dinitrophenol paralyzed oxidative phosphorylation in a kidney homogenate in much the same way as those reagents affected the bacterial and yeast test systems. In presence of minute amounts of gramicidin or dinitrophenol, oxidation in the cyclophorase system proceeds if anything more rapidly than normal whereas the process involving removal of phosphate by esterification of glucose is either suppressed completely or greatly inhibited (cf. Table VIII).

It has been mentioned above that the oxidation of fatty acids, α-β-unsaturated acids or β-keto acids was a process which did not proceed unless simultaneously some member of the citric acid cycle was undergoing oxidation. This phenomenon has been referred to as the sparking phenomenon. Gramicidin and dinitrophenol have turned out to be invaluable reagents for the analysis of this sparking phenomenon which underlies the process of activation of fatty acids and their derivatives. In presence of these reagents the sparking effect on the oxidation of fatty acids, unsaturated acids or β-keto acids is abolished despite the fact that the oxidation of the sparking substance remains unaffected (cf. Table IX). This result has interesting implications. It is not the oxidation of the sparker as such which underlies the sparking phenomenon but rather the phosphate transformation which accompanies the oxidation. The oxidation of the sparker leads to the esterification of inorganic phosphate and the formation of the primary ester. The sparking phenomenon presumably involves the reaction of the primary ester with the substance which has to be activated. According to this formulation, activation is equivalent to the formation of a complex between the coenzyme pyrophosphate (primary ester) and the substance to be activated. This complex then undergoes oxidation. Gramicidin and dinitrophenol can either inhibit the formation of the coenzyme pyrophosphate or the reaction of the coenzyme pyrophosphate with the substance to be activated.

In the light of the postulated mechanism of action of dinitrophenol and gramicidin it should follow that any oxidation process which requires sparking should be inhibited by these reagents and conversely any process which is sensitive to these reagents must involve activation of the substrate. Thus far five processes have been shown to require sparking and to be sensitive to these reagents. They are: (1) oxidation of fatty acid to

TABLE VIII

Inhibition of Phosphate Esterification by Gramicidin and the Dinitrophenol (cf. Reference 44)

	Concentration of gramicidin	O_2 uptake	P esterified	P/O ratio
	μg./cc.	μ atoms	μ moles	
1.	0	19.5	44.8	2.30
	10	20.7	14.0	0.68
	20	20.7	11.3	0.55
	40	22.3	14.5	0.65
	80	21.6	12.6	0.59
	Concentration of dinitrophenol			
2.	0	18.9	43.0	2.3
	M /5,000	18.7	2.6	0.1
	M /15,000	17.2	1.9	0.1
	M /60,000	21.0	20.3	1.0
	M/120,000	20.2	31.0	1.5

The phosphorylating system contained 1 cc. kidney enzyme (R_3K), 0.03 cc. of M sodium fluoride, 0.2 cc. of M/50 magnesium sulfate, 0.3 cc. of M/100 adenylic acid, 0.3 cc. of M/10 α-ketoglutarate, 0.5 cc. of M/25 phosphate buffer of pH 7.2, 0.1 cc. of M. fructose, and 0.1 cc. of yeast hexokinase, 25° and oxygen in gas space. The gramicidin and dinitrophenol experiments were carried out at different times and with different enzyme preparations.

TABLE IX

Effect of Dinitrophenol and Gramicidin on Sparked Oxidation of Butyrate, Aceto-acetate and Acetate (cf. Reference 44)

Reaction	Rate without inhibitor μ atoms oxygen	Rate with gramicidin 10 ug./cc. μ atoms oxygen	Rate with dinitrophenol M/15,000 μ atoms oxygen
Oxidation of butyrate	315	86	0
	206	28	17
Oxidation of acetoacetate	170	0	0
Oxidation of acetate	512	54	0

The system in each case consisted of 1 cc. enzyme (R_3K), 10 μ moles of substrate, 3 μ moles of α-ketoglutarate as sparker, 0.2 cc. of M/50 magnesium sulfate, 0.3 cc. of M/100 adenylic and 0.2 cc. of M/8 phosphate buffer of pH 7.2. The rates in the table have been corrected for the appropriate blank without added butyrate, acetoacetate and acetate; 25°, and oxygen in gas space.

unsaturated acid, (2) oxidation of unsaturated acid to keto acid, (3) the cleavage of β-keto acid into acetic acid and the residue fatty acid, (4) the condensation of acetic acid with oxaloacetic acid to form citric acid, and (5) the condensation of acetoacetic acid with oxaloacetic acid to form citric acid. Reaction (5) may well be a combination of reactions (3) and (4). The oxidative condensation of pyruvic acid and oxaloacetic acid to form citric acid is insensitive to both gramicidin and dinitrophenol, and consistent with this insensitivity is the fact that the oxidation of procitric to citric acid involves esterification of three moles of phosphate per atom of oxygen absorbed. The oxidative condensation of pyruvic acid to acetoacetic similarly is insensitive to the two reagents and also is accompanied by phosphate esterification (P/O ratio of 2).

If the linkage of phosphate esterification with oxidation is an obligatory one in the sense that oxidation cannot proceed without esterification of inorganic phosphate then it should follow that all the reactions catalyzed by the cyclophorase system should not proceed in the total absence of inorganic phosphate. The experimental verification of this corollary is complicated by the fact that even in absence of added inorganic phosphate there is a constant liberation of inorganic phosphate formed by dephosphorylation of various phosphoric esters in the protein of the cyclophorase complex and the rate of liberation of inorganic phosphate is sufficiently high that it becomes impossible to attain a zero level of phosphate ions. Figure 4 shows how the rate of oxygen uptake varies with the concentration of inorganic phosphate. One device to lower the concentration of phosphate ions to a level where the availability of phosphate ions becomes the limiting factor is that of using a phosphate acceptor system like the hexokinase-glucose system and trapping the inorganic phosphate in the form of sugar phosphate. But even under these conditions it has never been found possible to reduce the level of inorganic phosphate below 0.3 μ moles per cc. In Table X data are presented to show that phosphate ions are necessary for each of the oxidation reactions catalyzed by the cyclophorase system, in fact for all oxidation reactions which are linked with esterification of inorganic phosphate.

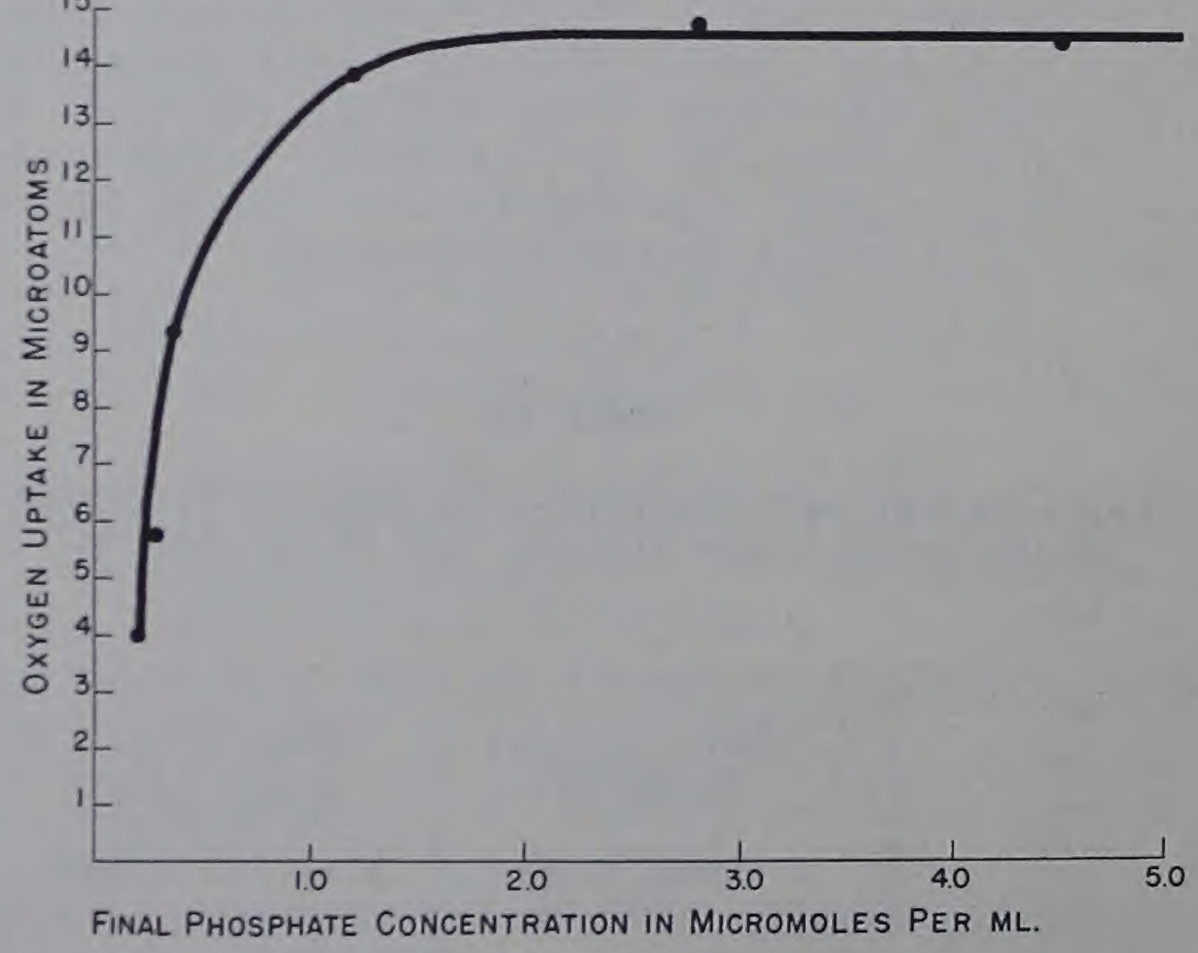


Figure 4

Phosphate requirement for α-ketoglutarate oxidation. Each manometer vessel contained 1 ml. of R_3K, 0.3 ml. of 0.01 M adenosine-5-phosphate, 0.2 ml. of 0.1 M magnesium chloride, 0.1 ml. of 0.1 M fluoride, 0.3 ml. of 0.1 M α-ketoglutarate, 0.9 per cent potassium chloride to a final volume of 2.8 ml., and oxygen in the gas space. In addition, the following amounts of inorganic phosphate of pH 7.3 were added to successive cups: 0, 3, 6, 10, 15, and 20 micromoles. The abscissa represents the final inorganic phosphate concentration. The ordinate represents oxygen uptake in the first 15 minutes after a 5 minute equilibration period at 25°.

TABLE X

Phosphate Requirements of Different Substrates
(cf. Reference 44)

Substrate	No hexokinase or sugar		With hexokinase and sugar	
	Oxygen uptake	Final phosphate concentration	Oxygen uptake	Final phosphate concentration
	c.mm.^{3}/10 min.	μM/cc.	c.mm.^{3}/10 min.	μM/cc.
α-Ketoglutarate	135	3.9	73	0.37
Succinate	136	5.0	88	0.40
Malate	102	4.3	54	0.40
Oxalacetate	94	2.9	52	0.47
Citrate	138	7.2	74	0.20
Glutamate	110	8.0	65	0.28

The experimental manometer vessels contained 1 cc. of the third residue from rabbit kidney, 0.5 cc. of 0.04 M phosphate buffer of pH 7.3, 0.3 cc. of 0.01 M adenosine-5-phosphate 0.2 cc. of 0.1 M. magnesium chloride, 0.1 cc. of 0.33 M sodium fluoride, 0.3 c. of 0.1 M substrate solution (except in the case of succinate when 0.2 cc. of 0.5 M solution was used), water to a final volume of 3.0 cc., alkali in the center well, and oxygen in the gas space. Each substrate was set up in duplicate, one cup containing in addition to the above components 0.02 cc. of yeast hexokinase preparation and 0.1 cc. of 1.0 M fructose. In the case of citrate and glutamate 30 micromoles of phosphate buffer were added instead of 20. All experiments were done at 25°. The oxygen uptake figures for the first three substrates listed represent the uptake during the last 10 minutes of a 30 minute experiment. For the others the average uptake is given for the whole experiment, which lasted 30 minutes with oxalacetate and 45 minutes with the other two.

Loomis and Lipmann (57) have made the interesting observation that in presence of dinitrophenol the oxidations of the cyclophorase system appear not to require inorganic phosphate and they suggest that dinitrophenol by "replacing" inorganic phosphate can break the link between oxidation and phosphorylation.

Since the oxidation reactions catalyzed by the cyclophorase system involve esterification of inorganic phosphate, it might be anticipated that all these reactions would involve a common mechanism and would depend upon the same coenzymes. As yet only adenylic acid has been shown to be essential for all but one of the oxidative enzymes of the cyclophorase complex. The succinic enzyme appears not to require adenylic acid under conditions where the other enzymes are inactive without supplementation.

Assuming that adenylic acid as such exists in combination with the protein moieties of various enzymes in the cyclophorase and that the autolytic process involves the splitting off of adenylic acid followed by its degradation then it may well be that all the oxidative enzymes require adenylic acid, but only for certain ones is the linkage with adenylic acid susceptible to attack by the autolytic processes at work. The term, adenyloproteins, has been adopted to describe those oxidases present in the cyclophorase system which can be shown to require added adenylic acid for activity. It may well be that all the oxidases of the cyclophorase complex will eventually be characterized as adenyloproteins since it appears that adenylic acid is probably the key coenzyme in the linkage of oxidation with phosphate esterification.

There may arise some confusion between the two roles in which adenylic acid is involved in the cyclophorase system and it is in order to consider each in turn. A freshly prepared kidney enzyme will catalyze the oxidation of α-ketoglutarate for example without

added adenylic acid. If, however, a hexokinase-glucose acceptor system is introduced then little or no phosphorylation of glucose will ensue unless adenylic acid is added. In this case adenylic acid is acting as a shuttle between the cyclophorase donor system on the one hand and the glucose acceptor system on the other. Thus adenylic acid serves in two capacities: (1) that of restoring the activity of the oxidases from which adenylic acid has been split off and (2) that of acting as a phosphate transporter between the phosphate donor and acceptor systems. In order to serve in the first capacity presumably adenylic acid has to combine with the apoenzyme of the oxidase whereas in the second capacity adenylic acid exists free in solution as such.

Adenylic acid can be replaced quantitatively by either adenosine triphosphate or adenosine diphosphate. All three forms are probably interconvertible in the enzyme complex. Inosinic acid and a new type of adenosine triphosphate isolated by Albaum from plants (58) can replace adenylic acid as coenzyme for the apooxidases. Adenosine, guanosine, guanylic and yeast adenylic are completely inactive in that capacity.

Cozymase can replace adenylic acid but there is no summation when both cozymase and adenylic acid are present. Alkali inactivated cozymase has the same effect as cozymase, a fact which suggests that cozymase is active only by virtue of its ability to undergo cleavage to form adenylic acid in presence of the cyclophorase system. Consistent with this interpretation is the observation that in exhaustively washed preparations of the cyclophorase system (e.g., at the R_5K stage) cozymase cannot replace adenylic acid. Presumably the enzyme responsible for its conversion to adenylic acid is either no longer present or has been inactivated. In that connection Kornberg (59a) has obtained from kidney cortex a pyrophosphatase which cleaves various nucleotides including cozymase at the pyrophosphate linkage. Adenylic acid is formed as an hydrolysis product of cozymase. Coenzyme II has no action whatsoever when tested as a substitute for adenylic acid. The same inactivity applies to other coenzymes such as pyridoxal phosphate and no direct evidence has been obtained that any added component other than adenylic acid or either of its two products of phosphorylation is essential for any of the oxidative reactions of fresh cyclophorase preparations. In the cyclophorase complex there are oxidases which catalyze the oxidation of malate, β-hydroxybutyrate, glutamate and citrate. There are pyridinoprotein enzymes known (60 - 63) which can catalyze the oxidation of each of these four substrates. May it be inferred that the equivalent enzymes in the cyclophorase complex are really the same pyridinoprotein enzymes but in a different physical state? This has been a particularly difficult point to settle with any finality and it would not be possible to examine all the data on which the decision has been based. However, the evidence may be summarized as follows. The cyclophorase enzymes which catalyze the oxidation of malate, β-hydroxybutyrate, citrate and glutamate appear not to require the presence of added pyridine nucleotides. Furthermore all these four cyclophorase oxidases catalyze reactions in which inorganic phosphate is esterified whereas the classical pyridinoproteins do not exhibit any such property. Finally, the cyclophorase oxidases all require the presence of inorganic phosphate and two of them (malate and glutamate) have been shown to require the presence of adenylic acid. The corresponding pyridinoproteins show no need for inorganic phosphate or for adenylic acid. On the face of it there would appear to be no basis for contending that any of the constituent enzymes of the cyclophorase are classical pyridinoprotein enzymes. There is one curious observation, however, which places the problem in a somewhat new light. There is present in the well washed cyclophorase enzymes obtained from both kidney and liver a very considerable amount of cozymase and coenzyme II which resist tenaciously all attempts to wash them out. There is every indication that these coenzymes are firmly bound to protein and are not freely diffusible. In the light of the consideration mentioned previously it cannot be maintained that the citric, malic, β-hydroxybutyrate and glutamate enzymes are classical pyridinoproteins. But the possibility is open that a new type of pyridinoprotein enzyme exists which involves three new characteristics: (1) rigid combination of the pyridine nucleotide with the enzyme, (2) dependence of the overall reaction upon the presence of inorganic phosphate and adenylic acid and (3) utilization of the pyridine nucleotide for the dual role of oxidation-reduction and phosphate esterification. However, it has yet to be shown that bound cozymase and coenzyme II are in any way connected with the activity of the cyclophorase enzymes.

Since this chapter was written evidence has now accumulated to show that the principal oxidases of the cyclophorase complex are indeed conjugated pyridinoproteins with properties which sharply distinguish them from those of the corresponding classical enzymes.

The cyclophorase complex contains two other enzymes, viz. the succinic and α-ketoglutaric oxidases which can be shown not to be identical with their classical counterparts. Thus the cyclophorase succinic enzyme requires inorganic phosphate and catalyzes a phosphate linked oxidation. Its classical counterpart (cf. Battelli & Stern (64)), Thunberg (65)) does not require inorganic phosphate and cannot catalyze a phosphate linked oxidation. Similarly the cyclophorase α-ketoglutaric oxidase is a typical adenyloprotein enzyme whereas its classical counterpart (66) is a diphosphothiaminoprotein enzyme which acts independent of the presence of inorganic phosphate or adenylic acid. In short, enough evidence is to hand to confirm that all the oxidation enzymes associated with the cyclophorase complex have properties which distinguish them from their classical counterparts. It remains to be determined how profound this distinction may be. Are we dealing with totally different enzymes or is the cyclophorase enzyme merely a variant of the classical type?

At various points reference has been made to the formation of a primary ester or coenzyme pyrophosphate as the first product of the esterification of inorganic phosphate. It is proposed to summarize in this section all the available evidence supporting this postulate and to clarify more precisely just what is implied by the terms primary ester and coenzyme pyrophosphate. In essence the assumption is made that the prosthetic groups of all the oxidative enzymes of the cyclophorase complex are the instruments by which inorganic phosphate becomes esterified. As the various prosthetic groups undergo reduction they simultaneously pick up a molecule of inorganic phosphate by an as yet unspecified mechanism. Furthermore, these prosthetic groups are firmly linked with the protein and cannot be readily split off by repeated washing of the cyclophorase gel.

Considerable amounts of coenzymes firmly bound to protein in the cyclophorase gel have been found (67). Thus far the presence of six different coenzymes in considerable amounts has been established: (1) adenosine triphosphate and adenylic acid, (2) cozymase, (3) flavinadenine dinucleotide, (4) diphosphothiamine, (5) the acetylation coenzyme of Lipmann (68) and Nachmansohn (69), and (6) triphosphopyridine nucleotide. In the light of the requirement of many of the oxidative enzymes of the cyclophorase system for adenylic acid it is highly significant that both adenylic acid and its phosphorylation product should be found present in the cyclophorase gel in a bound form. It is also very significant that an aged enzyme preparation which no longer is active without added adenylic acid discloses much smaller amounts of adenylic acid still bound to the protein than the corresponding fresh enzyme. The aging process seems to be accompanied by a parallel loss of bound adenylic acid.

No mention has been made thus far of the possibility that phosphate esterification in the cyclophorase system may be linked with the oxidation of the substrate leading to the formation of an acylphosphate or some similar phosphate complex. The possibility of forming phosphorylated intermediates has been explored by various investigators with consistently negative results (70). There is no evidence of a direct nature that any substrate undergoes oxidative phosphorylation in the cyclophorase complex. There is also no worthy evidence that acetyl phosphate or any other acyl phosphate is formed in the cyclophorase system or is capable of being oxidized as such by the cyclophorase system. Considering that certain oxidation reactions involve a P/O ratio of 3 it must be obvious that the phosphorylation of the substrate could account for only one of the three phosphates and the principle of coenzyme phosphorylation must be invoked to account for a ratio of 3. If three coenzymes operate in series and the reduction of each one of them is accompanied by esterification of inorganic phosphate then it would be possible to attain the P/O value of 3.

It is of considerable interest from the standpoint of comparative biochemistry to note that *Clostridium kluyveri* duplicates some of the key processes catalyzed by the cyclophorase system whilst employing a completely different mechanism of oxidative phosphorylation. Stadtman and Barker (71) have shown that caproate can be synthesized from ethyl alcohol by the following series of reactions:

$$(1)\quad CH_3CH_2OH \xrightarrow{-2H} CH_3CHO$$

$$(2)\quad CH_3CHO \xrightarrow[H_3PO_4]{-2H} CH_3CO\ PO_3H_2$$

There is reason to believe that the next reaction is the formation of acetoacetate by the condensation of acetylphosphate and acetate,

$$CH_3CO\ PO_3H_2 + CH_3COOH \longrightarrow CH_3CO\ CH_2COOH + H_3PO_4$$

although Stadtman and Barker have only demonstrated the reverse reaction, that is, the phosphoryclastic decomposition of acetoacetic acid. Presumably caproate is synthesized by reduction of acetoacetate to butyrate and repeating the condensation with acetylphosphate. The significant point of this very beautiful study is that only 1 mole of phosphate is taken up for each atom of oxygen absorbed during the oxidation of acetaldehyde in contrast to ratios of 3 for the oxidative steps of the cyclophorase system. Where the substrate is the instrument of oxidative phosphorylation the maximum ratio for P/O is 1.0. It is only with a coenzyme mechanism that ratios above 1 can be attained.

It would be idle to speculate on the possible mechanisms by which phosphate esterification would accompany oxidation-reduction in the cyclophorase system until there is precise evidence as to the chemical nature of the one or more primary esters or coenzyme pyrophosphates. Some important problems such as the mechanism of transphosphorylation between the primary ester and adenylic acid, the origin of inorganic pyrophosphate and the mechanism of action of dinitrophenol and gramicidin will have to be tabled until the structure of these coenzymes becomes known.

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Chapter XI

ENZYMATIC SYSTEMS CONCERNED WITH THE METABOLISM OF CERTAIN NITROGEN COMPOUNDS

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ENZYMATIC SYSTEMS CONCERNED WITH THE METABOLISM OF CERTAIN NITROGEN COMPOUNDS

While it is impossible to draw a sharp line which divides the respiratory enzymes from other metabolically important systems, it was nevertheless decided to draw such a line arbitrarily. Emphasis in this chapter has been placed on oxidative systems. However, since amino acids in particular undergo many important reactions which are not, strictly speaking, oxidative in character, reference is made to all types of reactions in which amino acids participate, exclusive of proteolytic systems. This has necessitated the exclusion of systems dealing with amino purines, certain amides, etc. It is hoped that whatever the disadvantages of a somewhat restricted scope, these will be overcome by the advantages of a review dealing with a more closely integrated subject.

THE AMINO ACID DEHYDROGENASES

The enzymes to be considered in this section are those which oxidatively deaminate amino acids. The type reaction is:

$$(1)\quad \underset{\underset{NHR''}{|}}{R'\text{-}CH\text{-}COOH} \rightleftharpoons \underset{\underset{NR''}{\|}}{R'\text{-}C\text{-}COOH} + 2H\cdot$$

followed by the spontaneous breakdown of the imino acid:

$$(2)\quad \underset{\underset{NR''}{\|}}{R'\text{-}C\text{-}COOH} \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \underset{\underset{O}{\|}}{R'\text{-}C\text{-}COOH} + R''NH_2$$

In the following discussion, the over-all reaction forming the keto acid, the amine (or ammonia) and two hydrogen atoms will be considered without representing the formation of the imino intermediate. The various enzymes differ only in the nature of the hydrogen acceptor necessary, and the specificity toward the various amino acids. In the usual case, R" is a hydrogen atom, and one of the products is ammonia. In the case of such compounds as N-methyl leucine, one of the products would be methylamine, etc. Proline and hydroxyproline present special cases, where R" is one end of the carbon chain, and oxidative "deamination" of these compounds results in the formation of δ-amino α-ketovaleric acids (Krebs, 1939).

The student of amino acid metabolism, at least in the case of the mammalian tissues, is presented with the anomalous situation of having comparatively detailed information concerning enzymes whose physiological function is of questionable significance and being virtually ignorant of the nature of those enzymes which might be expected to play a more important role. Those interested in having confusion presented in tabulated form should consult the summary of the existing knowledge concerning L-amino acid metabolism by Edlbacher and Grauer (1944). At present, two anaerobic dehydrogenases have been unequivocally demonstrated, the L-amino acid dehydrogenase from Proteus vulgaris, and L-glutamic dehydrogenase from plants and animals. Four aerobic dehydrogenases are known: the ophio L-amino acid dehydrogenase, and the L-amino acid dehydrogenase, glycine dehydrogenase, and D-amino acid dehydrogenase from mammals. Considering for the moment only mammalian metabolism, the list, containing four enzymes from mammalian sources, would seem to permit explanation of the general breakdown of amino acids. However, the L-amino acid dehydrogenase which has been characterized is not sufficiently active to explain the observed rate of breakdown of the L-compounds in tissue slices.

A further line of demarcation which can be made in classifying the oxidative enzymes is that of cyanide sensitivity. Those enzymes whose activity is not dependent upon metalloproteins, i.e., the flavin and pyridine nucleotide enzymes, will function in the presence of cyanide ion, while those dependent upon metalloproteins will not. It should be noted

that the primary oxidizing enzyme need not be a metalloprotein; it may require a metalloprotein hydrogen transport system such as the cytochrome system. Of all the characterized enzymes, only that from Proteus vulgaris is inhibited by cyanide. On the other hand, Krebs' original "L-amino acid dehydrogenase" demonstrated in tissue slices and concentrated liver and kidney brei (Krebs, 1933, 1935a) was sensitive to this agent, and must, if it is a single entity, differ from the aerobic dehydrogenase mentioned above. Other claims have been brought forward for the existence of as yet uncharacterized histidine, proline, and phenylalanine dehydrogenases (Edlbacher and Grauer, 1944). Until more is known concerning the nature of these enzymes, little can be said concerning them. Most of the oxidations observed with L-amino acids by cyanide insensitive tissue extracts could possibly be explained by the L-amino acid dehydrogenase now characterized. The cyanide sensitive reactions may be catalyzed by metalloproteins, by pyridine nucleotide proteins linked to the cytochrome system, or even conceivably by systems not sensitive in themselves to cyanide, but coupled to cyanide sensitive reactions.

It is plain that no detailed picture of nitrogen metabolism in terms of specific enzymatic reactions can be drawn. In the tissue slice, tissue brei, and crude tissue extract, the breakdown of a particular amino acid may invoke transamination, transimination, decarboxylation, and possibly other yet unknown non-oxidative reactions yielding different nitrogen compounds more susceptible to attack, so that a direct interpretation of the results obtained with such systems is usually difficult and subject to many doubts. The great need of the field is a comprehensive study, under conditions where the complex metabolic interplay of the intact tissue is eliminated.

The detailed consideration of the known amino acid dehydrogenases that follows is offered then, with the reservation that the presentation covers only a fragment of the enzymes involved in the oxidative breakdown of amino acids.

AEROBIC DEHYDROGENASES

The hydrogen acceptor for this group of enzymes is molecular oxygen, and the type reaction is:

$$(3)\quad 2H\cdot + O_2 \longrightarrow H_2O_2$$

In the absence of catalase activity, the peroxide formed may react with the keto acid in varying amounts:

$$(4)\quad R'\text{-}\underset{\underset{O}{\|}}{C}\text{-}COOH + H_2O_2 \longrightarrow R'\text{-}COOH + CO_2 + H_2O$$

so that the over-all reaction approaches a summation of (1), (2), (3) and (4):

$$(5)\quad R'\text{-}\underset{\underset{NHR''}{|}}{CH}\text{-}COOH + O_2 \longrightarrow R'\text{-}COOH + R''NH_2 + CO_2$$

DL-Proline has been reported as an exception where the L-amino acid is more subject to attack by hydrogen peroxide than is the δ-amino α-ketovaleric acid resulting from the oxidation of the D-acid (Klein and Handler, 1941).

In the presence of catalase which decomposes the hydrogen peroxide:

$$(6)\quad H_2O_2 \longrightarrow H_2O + \tfrac{1}{2}O_2$$

the over-all reaction is:

$$(7)\quad R'\text{-}\underset{\underset{NHR''}{|}}{CH}\text{-}COOH + \tfrac{1}{2}O_2 \longrightarrow R'\text{-}\underset{\underset{O}{\|}}{C}\text{-}COOH + NH_2R''$$

Keilin and Hartree (1936) devised a system offering an excellent method for demonstrating hydrogen peroxide formation (and removing it before it can attack other substrates or be decomposed by catalase) involving a coupled oxidation of alcohol:

$$(8)\quad CH_3CH_2OH + H_2O_2 \xrightarrow{\text{catalase}} CH_3CHO + 2H_2O$$

so that the total system follows equation

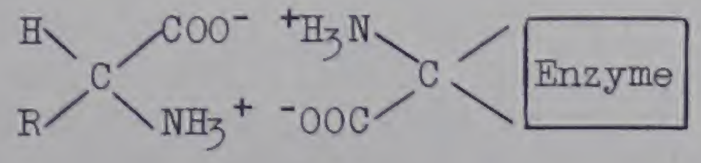
$$(9)\quad \underset{\underset{NHR''}{|}}{R'\text{-}CH\text{-}COOH} + O_2 + CH_3CH_2OH \longrightarrow \underset{\underset{O}{\|}}{R'\text{-}C\text{-}COOH} + R''NH_2 + CH_3CHO + H_2O$$

This system exhibits the theoretical oxygen uptake without destruction of the keto acid as in equation (5).

L-Amino Acid Dehydrogenases

Zeller and Maritz (1944) have described an enzyme originally discovered in snake venoms, and later shown to be present in various tissues of both venomous and non-venomous snakes, which was termed ophio L-amino acid oxidase (later shortened to the unfortunate expression "ophio oxidase"). Since the venoms contain no catalase, the reaction is (5) above. With added catalase, reaction (7) occurs and the keto acid can be demonstrated. The enzyme is freely soluble in water, stable to dialysis, inactivated at 100°, unaffected by KCN, NH_4, or capryl alcohol. While its mode of action would indicate that it is a flavoprotein, the nature of the prosthetic group is unknown.

The enzyme has a sharp optimum at pH 7.2 (leucine substrate) and has an optimum substrate concentration (6.8×10^{-3} M leucine). Zeller and Maritz (1944) interpret this as being due to the formation of a substrate-enzyme complex of the type:



and point out that those sulfonic acids forming difficultly soluble salts with amino groups should inhibit, which they do. For example, 0.002 M 3-nitro 2-toluenesulfonic acid inhibits 50% (Zeller, Iselin and Maritz, 1946). Various sulfonic acids of this type inhibit 50-80% at 2.9×10^{-3} M and 90-98% at 2.9×10^{-2} M (Zeller and Maritz, 1944). However, they neglect to mention that any reagent which removes the enzyme or substrate from solution, or which combines with the amino or carbonyl groups of the substrate could be expected to be inhibitory regardless of the nature of the enzyme-substrate linkages.

The enzyme is also inhibited by benzoate and iodoacetate (Zeller, Iselin and Maritz, 1946), and competitively by carbonyl reagents (Zeller and Maritz, 1945d).

The specificity varies from species to species (Zeller, Iselin and Maritz, 1944), although the general pattern is the same. The rates for Vipera aspis venom are summarized in Table 1 (Zeller and Maritz, 1944).

TABLE I

Amino Acid	Q_{O_2}	Amino Acid	Q_{O_2}
Phenylalanine	780	Diiodotyrosine	135
Leucine	612	Nitrotyrosine	129
Tryptophane	540	Histidine	78
Tyrosine	402	Valine	71
Methionine	390	Cystine	42
Dihydroxyphenylalanine	294	Alanine	30
Dibromtyrosine	216	Arginine	26
Isoleucine	162	Cysteine	22

Citrulline, serine, ε-benzoyllysine, asparagine, and glutamine are also oxidized (Zeller and Maritz, 1945c, d). The dicarboxylic acids, and proline, hydroxyproline, lysine, ornithine, N-methylleucine, glycine, sarcosine, hippuric acid, β-alanine, glycylleucine, glycyltyrosine, leucylglycine, and all D-amino acids are not attacked (Zeller and Maritz, 1944). Zeller and Maritz (1945a) have taken advantage of the high activity and specificity of the enzyme to adapt it to a manometric measurement of peptidase activity, which should be a most useful tool.

In contrast to the snake L-amino acid dehydrogenase, Green's laboratory has reported (Blanchard, Green, Nocito and Ratner, 1944, 1945; Green, Moore, Nocito and Ratner, 1944; Green, Nocito and Ratner, 1943) the preparation of a mammalian enzyme in purified form which has a strikingly low order of activity. The reactions catalyzed are the same as those of the ophio L-amino acid dehydrogenase.

The enzyme was prepared from rat kidney by salt fractionation procedures, 5 kilograms of kidney yielding 1 gram of the purified protein. The material is homogeneous in the electrophoresis apparatus, but shows two components in the ultracentrifuge (Green, Moore, Nocito and Ratner, 1944). The lighter component has an approximate molecular weight of 120,000 and contains 2 flavinmonophosphate molecules per mole. The heavy component has a molecular weight in excess of 430,000, and is believed to be the tetramer of the light component with 8 flavinmonophosphates per mole. Both components have equal catalytic activity. The flavin may be removed by heat or acid (with attendant denaturation of the protein moiety). The purified protein is contaminated with small amounts of some iron porphyrin.

The enzyme has a pH optimum at 10.0, a Q_{O_2} of 52 (turnover number of 6) at 38° at pH 8.8 with leucine as substrate (Blanchard, Green, Nocito and Ratner, 1945). It contains no xanthine, aldehyde, D-amino acid or glycine dehydrogenases. Originally, diaphorase activity was thought to be associated with the same protein, but this was later shown to be L-hydroxy acid dehydrogenase activity which cannot be separated from the amino acid dehydrogenase and is regarded as being a second function of the same protein. The enzyme has a constant temperature coefficient from 10-60°, but is completely denatured in 5 minutes at 70° (Blanchard, Green, Nocito and Ratner, 1944). The enzyme is low or absent in cat, dog, guinea pig, rabbit, pig, ox, or sheep tissues, and the best source is rat liver or kidney. Its peculiarly narrow distribution alone would render its quantitative significance in mammalian metabolism suspect.

Relative specificities are listed in Table II (Blanchard, Green, Nocito and Ratner, 1944).

TABLE II

Amino Acid	Relative Rate	Amino Acid	Relative Rate
L- N-Methylleucine	115	DL-Phenylalanine	45
L- Leucine	100	L- Tryptophan	40
DL-α-Aminocaproic	89	DL-Valine	28
DL-Methionine	81	L- Histidine	9
L- Proline	77	DL-α-Aminobutyric	3
DL-α-Aminovaleric	53		

Norleucine, norvaline, tyrosine, cysteine, cystine, alanine, S-benzyl or ethylcysteine, homocysteine, S-benzylhomocysteine are also oxidized. Glycine, threonine, serine, N-dimethylleucine, α-aminoisobutyric acid, and the dibasic and diaminoacids are not oxidized.

The various inhibitor effects are summarized in Table III (Blanchard, Green, Nocito and Ratner, 1944).

TABLE III

Inhibitor	Molarity	% Inhibition	Comments
NH_4^+	0.012	69	
NH_4^+	0.066	96	
$CuSO_4$	0.001	43	-SH inhibition
Iodoacetate	0.001	71	-SH inhibition
Benzoate	0.001	13	probably competitive
KCN	0.002	0	
Capryl alcohol	satd.	0	

Glycine Dehydrogenase

Ratner, Nocito, and Green (1944) have partially purified an apoenzyme from pig kidney which, upon addition of flavin adenine dinucleotide, oxidizes glycine or sarcosine according to equation (7), since the preparation contains excess catalase.

The enzyme was prepared by salt precipitation from an aqueous extract of acetone powder. No quantitative information is given to enable calculation of yield, activity per weight of tissue, or protein content of the final preparation. However, the final preparation represents an activity such that the acetone powder should oxidize at least 5 micromoles of glycine per gram of powder per hour. As mentioned, the enzyme is resolved during the isolation, and activity can only be restored by flavin adenine dinucleotide (it is not stated whether flavin monophosphate was tested). While the preparation contained D-amino acid dehydrogenase, the identity of the two enzymes has been excluded by differences in the conditions of dissociation of the prosthetic group.

The enzyme is not affected in 3 minutes at 55°, but is 75% destroyed in 3 minutes at 60°. It has optimum activity at pH 8.3 and one-eighth activity at pH 7.0. The half-velocity substrate concentration is approximately 0.04 M. It has been found in the kidney and liver of all animals tested (cat, dog, lamb, ox, rat, pig, human, rabbit) except rat kidney.

Inhibitor studies are summarized in Table V (Ratner, Nocito and Green, 1944).

TABLE IV

Inhibitor	Molarity	% Inhibition	Comments
Iodoacetate	0.01	30	-SH sensitive
"	0.001	0	
$CuSO_4$	0.001	100	" "
"	0.0002	64	" "
"	0.0001	37	" "
NaF	0.2	0	
$ZnSO_4$	0.001	0	
CN^-	0.01	0	
Sulfadiazine	0.005	0	
Capryl alcohol	satd.	0	

The enzyme does not attack N-dimethylglycine, phenylglycine, p-aminophenylglycine, creatine, or peptides of glycine.

D-Amino Acid Dehydrogenase

The existence in the liver and kidney of mammals of a strikingly active enzyme catalyzing the oxidation of the unnatural amino acids was first demonstrated by Krebs (1933), who isolated the α-ketoacids formed in an arsenite poisoned system. The further extension of this study (Bernheim and Bernheim, 1935; Bernheim, Bernheim and Gillaspie, 1936) excited considerable interest with the result that this enzyme is one of the most thoroughly investigated of all oxidative enzymes. While its physiological role is still in doubt, its ubiquitous occurrence suggests that either D-amino acids may be formed in the mammalian cell, or that any racemization which might occur, enzymatically or otherwise, would present the organism with such a toxic or inhibitory substance that it is essential to remove it immediately (it should be noted that certain peptides containing D-amino acids are potent antibiotics, e.g., Gramicidin S).

Negelein and Brömel (1939) prepared the purified apoenzyme from sheep kidney in 4.2% yield by resolving the enzyme with HCl, and alternating removal of impurities by warming at pH 5.1 and precipitation with 0.21 saturated ammonium sulfate. Warburg and Christian (1938); Warburg, Christian and Griese, (1938) isolated and characterized the flavin adenine dinucleotide prosthetic group.

One gram molecule of the flavin binds 100,000 grams of the purified protein (Negelein and Brömel, 1939). Negelein and Brömel assume by analogy with other flavoproteins that the molecular weight of the pure protein is about 70,000, and that their preparation is approximately 70% pure. The turnover number of the pure protein is estimated at about 2000 (D-alanine) by these authors, while Warburg and Christian (1938) estimate the turnover to be 1440 per mole of flavin per minute. If the protein had a molecular weight of 100,000 and the preparation were nearly 100% pure, the turnover number of Negelein and Brömel would be 1,400, or close to that obtained by Warburg. Assuming the 1,440 value to be correct, the protein would be about 97% pure.

Of interest is the demonstration that under anaerobic conditions only the flavin equivalent to the added protein is reduced concomitantly with the oxidation of amino acids indicating that the prosthetic group does not dissociate, and there is no formation of hydrogen peroxide (Negelein and Brömel, 1939). Upon exposure of the reduced flavoprotein to oxygen, the flavin is reoxidized with the formation of hydrogen peroxide. This confirms the expectation that the flavin moiety is the primary oxidizing reagent in the reaction.

Stadie and Zapp (1943) have presented a theoretical treatment of the kinetics of coenzyme linked reactions, and have found that D-amino acid dehydrogenase has identical affinity within experimental error for the substrate (D-alanine) when the enzyme is in either the zwitter or anion forms, the respective values for K_s being 0.0087 and 0.0092 M. Other estimates of K_s are 5, 6.1-6.6 M (Hellerman, Lindsay and Bovarnick, 1946; Keilin and Hartree, 1936). The $K_{flavin\text{-}protein}$ is of the order of 2.5×10^{-7} M (Hellerman, Lindsay and Bovarnick, 1946: Negelein and Brömel, 1939; Stadie and Zapp, 1943). Stadie and Zapp (1943) found that the flavoprotein may be treated as an acid with a pK of 7.9, so at pH 7.9 and 0.009 M alanine, the enzyme is half-dissociated as an acid, and both the forms are half-combined with the substrate.

The specificity of the oxidation has been investigated in many laboratories. The most thorough study with the purified enzyme is that of Klein and Handler (1941). The major criticism of their summary is that the relative specificity was determined by allowing the reaction to come to a halt and calculating the amount of amino acid oxidized at this point. Thus, the values obtained represent the rate of oxidation during the time before complete inactivation of the enzyme. Assuming the enzyme to be inactivated at the same rate, regardless of substrate, the values become true relative rates of oxidation, but this assumption is doubtful (cf. Edlbacher below). Further, the different substrates were not added in the same concentration. However, the data give a clear picture of the amino acids attacked and a rough estimate of their relative effectiveness as substrates. The tabulated data were recalculated to give a relative picture in terms of quantity of amino acid oxidized (D-alanine = 100) rather than the percentage of added amino acid oxidized, as presented in the original paper (Klein and Handler, 1941).

TABLE V

Amino Acid	Relative Oxidn.	Amino Acid	Relative Oxidn.
D-Alanine	100	DL-Phenylglycine	11
DL-α-Aminobutyric	81	DL-Tryptophan	10
D-Valine	76	D-Histidine	8
DL-Norvaline	75	DL-Serine	8
D-Isoleucine	69	DL-Arginine	8
DL-Norleucine	68	L-Threonine	7
DL-Proline	64	DL-Glutamic	0
D-Leucine	64	DL-Lysine	0
DL-Methionine	58	DL-Pseudoleucine	0
D-Phenylalanine	50	DL-Cystine	0
DL-Tyrosine	31	Glycine	0
DL-Aspartic Acid	17	β-Alanine	0
l-Allothreonine	13	α-Aminoisobutyric	0

Other investigators report the oxidation of α-amino β, γ-dihydroxybutyric acid, S-ethylcysteine, dihydroxyphenylalanine, ornithine, N-methylalanine, and pyrroline carboxylic acid. N-methyltyrosine, N-dimethylalanine, and β-hydroxyglutamic acid are not affected (Keilin and Hartree, 1936; Krebs, 1935a).

Edlbacher and Wiss (1945a, b; Edlbacher, Wiss and Walser, 1946) note an anomalous stimulation of dilute solutions of the enzyme by various amino acids, especially histidine, by two proteins removed during the purification, or by HCN or pyrophosphate (0.001 M HCN increased the rate of breakdown of 0.02 M D-alanine by 1.5 mg. of the purified protein 100 fold). The action is suggestive of a protection against metal catalyzed -SH autooxidation, but the point has not been settled.

The action of various inhibitors is summarized in Table VI.

TABLE VI

Inhibitor	Molarity	% Inhibition	Author	Comments
NaCl	0.1	25	Krebs, 1935a	
"	1.0	85	" "	
Capryl alcohol	satd.	0	" "	
NaCN	0.1	0	" "	
Ethyl urethane	0.57	100	Keilin and Hartree, 1936	
" "	0.28	70	" " "	
" "	0.14	24	" " "	
" "	0.057	12	" " "	
Phenyl "	0.003	0	" " "	
Pyrophosphate	0.02	0	" " "	
Sodium azide	0.01	0	" " "	pH 7.8
" "	0.002	53	" " "	pH 6.8
H_2S			" " "	inhibition by oxidation product - not inhibitory anaerobic.
Oxygen		variable	Bernheim, 1940	oxidation of SH group.
$CuSO_4$	0.00015	100	" "	see below.
Benzoic acid	0.0001	60 (alanine)	Klein and Kamin 1941	Varies with substrate.

TABLE VI (Continued)

Inhibitor	Molarity	% Inhibition	Author	Comments
p-Chlormercuri-benzoate	0.0001	78	Singer and Barron 1945	SH inactivation - relieved by glutathione.
Iodoacetamide	0.005	0	" " "	
p-Aminophenyl-dichlorarsine	0.00005	90	" " "	SH inactivation
Sulfanilamide	0.001	65	Hellerman, Lindsay and Bovarnick, 1946	1.1×10^{-7}M FAD competes with flavin
	0.01	2	" "	
Atabrine	0.001	70	" "	9.4×10^{-7}M FAD
	0.001	8	" "	0.4×10^{-7}M FAD competes with flavin
				27.5×10^{-7}M FAD

Bernheim (1940) claims the inhibitory action of cupric ion is not due to catalysis of autooxidation, but to direct combination. However, the enzyme is evidently quite sensitive to SH reagents. The benzoic acids are competitive with the substrate. Of great interest is the observation that such compounds as atabrine, quinine, plasmochin, sulfanilamide, aniline, the quinolines and auramines compete with flavin adenine dinucleotide for the apoenzyme (Hellerman, Lindsay and Bovarnick, 1946). Using a crude preparation of the enzyme, the $K_{\text{quinine-protein}}$ was calculated as $4.6\text{-}8.9 \times 10^{-4}$, as compared to a $K_{\text{FAD-protein}}$ of $4.6\text{-}6.9 \times 10^{-7}$ measured under the same conditions.

N-Methyl Amino Acid Dehydrogenase

Mention should be made of an enzyme reported by Ling and Tung* (1948) which oxidatively demethylates N-methyl amino acids to formaldehyde and the unsubstituted amino acid. The more detailed earlier papers were not available at this writing. However, it was stated that the enzyme has flavin adenine dinucleotide as the prosthetic group, so the reaction probably is:

$$H_2O + \text{R-CH(NHCH}_3\text{)-COOH} + O_2 \longrightarrow \text{R-CH(NH}_2\text{)-COOH} + HCHO + H_2O_2$$

The enzyme was reported to occur in the kidney and liver of various species, and to attack many N-methyl L-amino acids, but not N-dimethyl amino acids or derivatives of D-amino acids. A partial purification of an acetone powder extract was reported, but, if the above reaction is correct, the purified enzyme must contain catalase, since the amount of oxygen uptake reported was 0.5 to 0.6 that of the formaldehyde formed.

A more critical evaluation of this unique and interesting reaction must await detailed reports and independent confirmation.

ANAEROBIC DEHYDROGENASE

L-Amino Acid Dehydrogenase (Proteus Vulgaris)

Bernheim, Bernheim, and Webster (1935) first noted the cyanide-sensitive oxidation of L-amino acids by Proteus vulgaris. Stumpf and Green (1944) studied the reaction in cell-

*Tung also appears in the literature as Tai-sei To and Tatuo Yosida.

free extracts. A young culture of the organism oxidizes 22 amino acids. Upon aging, the number is reduced to 11, all of which are claimed to be broken down by a single enzyme. The aged cells were subjected to ultra-sonic disintegration, and the supernatant after centrifuging 1 hour at 2600 g. was employed. Further centrifugation at 100,000 g for 23 minutes brought down 58% of the material. The intact cells had a Q_{O_2} of 104, the cell-free extract 89, and the material sedimentable at 100,000 g, 290. The enzyme is thus associated with particulate material.

The nature of the enzyme is not known, and the reaction is (7), no hydrogen peroxide being produced. It is destroyed 78% at 55° and 100% at 60°, in 5 minutes exposure. The pH optimum is 6.8, K_S is 0.006 M, and the enzyme has a constant temperature coefficient from 30 to 50°. It reacts anaerobically with methylene blue.

The relative rates with various amino acids are summarized in Table VII (Stumpf and Green, 1944).

TABLE VII

Amino Acid	Relative Rate	Amino Acid	Relative Rate
DL-Norleucine	100	DL-Isoleucine	14
DL-Phenylalanine	93	DL-α-Aminobutyric	11
L-Leucine	84	DL-Serine	0
L-Typotophan	81	L-Aspartic Acid	0
L-Methionine	60	L-Glutamic Acid	0
L-Tyrosine	57	DL-Alanine	0
*DL-α-Aminocaproic	56	DL-Valine	0
DL-Norvaline	56	L-Proline	0
*DL-α-Aminovaleric	47	DL-Threonine	0
L-Histidine	31	L-Ornithine	0
*DL-α-Aminocaprylic	28	L-Lysine	0
L-Arginine	28	DL-Phenylglycine	0
*DL-α-Phenyl α-Aminobutyric	23	Glycine	0

The action of various inhibitors is summarized in Table VIII (Stumpf and Green, 1944).

TABLE VIII

Inhibitor	Molarity	Percent Inhibition	Comments
Ag^+	0.001	100	SH block (?)
Cu^{++}	0.001	62	" "
Hg^{++}	0.001	100	" "
Benzoate	0.01	0	
NaN_3	0.01	0	
CN^-	0.0033	76	
CN^-	0.00033	22	
AsO_3^{---}	0.001	0	
F^-	0.01	0	
Iodoacetate	0.01	0	
Capryl alcohol	satd.	100	

*Obtained with aged cell suspension. Remainder obtained with cell-free supernatant.

The enzyme has some interest in the resemblance to Krebs' (1933, 1935a) original L-amino acid dehydrogenase activity in mammals, particularly in regard to the sensitivity to cyanide and capryl alcohol. However, 0.01 M cyanide is not inhibitory in the presence of methylene blue, which indicates that the inhibition by cyanide is due to its effect on the cytochrome system, and that the enzyme itself transfers electrons to cytochrome. (An interesting speculative possibility is that the aerobic L-amino acid dehydrogenase of rat kidney is in actuality a similar enzyme, whose preferred electron acceptor is the cytochrome system, rather than molecular oxygen. If the enzyme were associated with cytochrome oxidase in a fashion similar to succinic dehydrogenase, the low turnover with molecular oxygen and the failure to obtain the enzyme from other species could possibly be explained.)

Based only on the observation of phenylalanine oxidation, the enzyme is believed to occur in various microorganisms (Stumpf and Green, 1944).

L-Glutamic Dehydrogenase

After the observation by Weil-Malherbe (1936) that the only amino acid attacked by brain is glutamic acid, the specific L-glutamic dehydrogenase was intensively studied by v. Euler's group and Dewan (Adler, 1937; Adler, Das, v. Euler and Heyman, 1937; Adler, Das and v. Euler, 1937; Adler, Günther and Everett, 1938; Dewan, 1938; v. Euler, Adler and Steenhoff-Eriksen, 1937; v. Euler, Adler, Günther and Das, 1938).

It is the only known pyridine nucleotide protein attacking an amino acid. The reaction is:

$$(10)\quad \begin{matrix}\mathrm{COOH}\\ |\\ (\mathrm{CH_2})_2\\ |\\ \mathrm{CHNH_2}\\ |\\ \mathrm{COOH}\end{matrix} + \mathrm{Pyr} + \mathrm{H_2O} \rightleftharpoons \begin{matrix}\mathrm{COOH}\\ |\\ (\mathrm{CH_2})_2\\ |\\ \mathrm{C{=}O}\\ |\\ \mathrm{COOH}\end{matrix} + \mathrm{NH_3} + \mathrm{Pyr\,H_2}$$

In plants and B. coli, diphosphopyridine nucleotide (DPN) is required; in yeast, triphosphopyridine nucleotide (TPN); and the animal enzyme will utilize either coenzyme (Adler, Günther and Everett, 1938; Mehler, Kornberg, Grisolia and Ochoa, 1947).

The usual preparation employed is an aqueous extract of liver acetone powder (Adler, Das, v. Euler and Heyman, 1937; Dewan, 1938). The reaction is reversible (Adler, 1937). The K_s is about 7.5×10^{-3} M (Adler, 1937), and the optimum is pH 8 (yeast enzyme) (v. Euler, Adler and Steenhoff-Eriksen, 1937). The enzyme is inhibited 100% by 0.0005 M p-chloromercuribenzoate or 0.001 M 3-amino 4-hydroxyphenyldichlorarsine, and therefore has SH groups essential for activity (Singer and Barron, 1945). Various dicarboxylic acids competitively inhibit (asparate inhibits 55% at 0.06 M) (v. Euler, Adler, Günther and Das, 1938).

The equilibrium constant for reaction (10) as written depends on the pH, becoming higher at higher pH values. For example, $K = 1 \times 10^{-3}$ at pH 8.05, or 5×10^{-6} at pH 6.05. In the absence of any system oxidizing reduced DPN, the reaction favored would be the reductive amination of α-ketoglutaric acid (v. Euler, Adler, Günther and Das, 1938).

Since the protein has not been obtained in a pure form, little more is known about the enzyme except that it is specific to L-glutamic acid.

MONOAMINE OXIDASE

(Amine dehydrogenase, Tyramine oxidase, Amine oxidase)

Monoamine oxidase(s) catalyzes the following general reaction (Hare, 1928; Pugh and Quastel, 1937; Philpot, 1937):

$$RCH_2NH_2 + O_2 \longrightarrow RCH = NH + H_2O_2$$

$$RCH = NH + H_2O \longrightarrow RCHO + NH_3$$

The reaction is usually followed by measuring O_2 uptake although NH_3 formation may also be determined. In the presence of aldehyde fixatives the aldehyde yield is practically quantitative (Richter, 1937); in the absence of these, a dismutation occurs to yield the corresponding acid and alcohol (Pugh and Quastel, 1937; Philpot, 1937; Richter, 1937). The manometric estimation is usually carried out in the presence of cyanide to which the system is insensitive. Surprisingly little progress has been made in purification of this enzyme system.

The substrate specificity has been studied by a number of investigators (Richter, 1937; Kohn, 1937; Blaschko, Richter and Schlossmann, 1937; Bhagvat, Blaschko and Richter, 1939; Beyer, 1941; Alles and Heegaard, 1943; Snyder, Goetze and Oberst, 1946; Snyder and Oberst, 1946). Blaschko, Richter and Schlossmann (1937) presented strong evidence to support the view that the previously reported tyramine oxidase (Hare, 1928; Philpot, 1937; Bernheim, 1931) epinephrine oxidase (Blaschko, Richter and Schlossmann, 1937a) and aliphatic amine oxidase (Pugh and Quastel, 1937) were one and the same enzyme. Thus the different amine oxidases were shown to be similar in respect to

1) the chemical reactions catalyzed
2) their distribution in the animal kingdom
3) the effect of inhibitors
4) the competition between the substrates

However, more recently, Alles and Heegaard (1943) using purified liver preparations from different species have reported that their data, in addition to that of others, make it questionable that a single enzyme is involved.

The effect of chemical structure on reactivity of amines may be summarized as follows (Blaschko, Richter and Schlossmann, 1937; Alles and Heegaard, 1943; Hartung, 1946; Beyer and Morrison, 1945):

1. Secondary methylamines of the type $R\text{-}CH_2\ NHCH_3$ including epinephrine, are oxidized to form methylamine in place of ammonia; tertiary amines, $RCH_2\text{-}N(CH_3)_2$, such as hordenine, react more slowly to form dimethyl amine.
2. Aliphatic amines are deaminated more rapidly as the hydrocarbon chain is lengthened; branched chain compounds of the type $RR\ CHCH_2NH_2$ react more slowly than the straight chain isomers.
3. Secondary carbinamines of the type $RR\ CH\ NH_2$ are completely inactive, but inhibit mono amine oxidase activity.
4. Diamines of the type $NH_2 - (CH_2)_7 - NH_2$ are not oxidized.
5. Substitution of the benzene ring in phenylethylamine results in slight to marked changes in deamination rate by monoamine oxidase, depending on the nature and position of the substituent group.

A comparative study of the oxidation of a series of amines by liver preparations from different species is shown in Table IX. Kinetics (Alles and Heegaard, 1943) and inhibitor specificity (Heegaard and Alles, 1943) of monoamine oxidase systems have been studied. Constants for enzyme-substrate compounds are shown in Table X, calculated from the Michaelis and Menten equation (1913) according to Lineweaver and Burk (1934).

TABLE IX

Liver Extract Oxidation; Maximum Oxidation Rates (Per Cent) Relative to Phenethylamine (Alles and Heegaard, 1943)

At 30° with 0.008 M amine substrates in phosphate buffer, pH 7.0.

	Rabbit	Guinea pig	Cat	Cattle
Ethylamine	0	0	20	5
Butylamine	50	100	90	110
Amylamine	110	140	110	85
Hexylamine	120	80	90	85
Heptylamine	130	70	100	85
Benzylamine	30	10	100	130
Phenethylamine	100	100	100	100
Phenpropylamine	110	30	90	105
Phenethanolamine	30	30	55	5
Phenethylmethylamine	105	120	105	65
3-Hydroxyphenethylamine	70	190	110	95
4-Hydroxyphenethylamine (tyramine)	90	200	120	130
4-Hydroxyphenethylmethylamine	65	160	80	85
4-Hydroxyphenethanolmethylamine (synephrine)	25	70	40	5
4-Hydroxyphenethyldimethylamine (hordenine)	30	10	45	80
3,4-Dihydroxyphenethylamine (hydroxytyramine)	65	200	80	85
3.4-Dihydroxyphenethylmethylamine (epinine)	65	180	30	65
3,4-Dihydroxyphenethanolamine (arterenol)	25	40	20	5
3,4-Dihydroxyphenethanolmethylamine (DL-epinephrine)	15	40	20	5
1-3,4-Dihydroxyphenethanolmethylamine (epinephrine)	25	40	20	5

TABLE X

Constants for Enzyme-Substrate Compounds (Alles and Heegaard, 1943)

Substrate	$\frac{1}{K_s}$	$\frac{1}{V_{max.}}$	K_s	$V_{max.}$
			M	microliters O_2 per 15 min.
Amylamine	1320	0.024	0.0008	42
Hexylamine	1000	0.017	0.0010	59
Heptylamine	750	0.011	0.0013	90
Octylamine	420	0.009	0.0024	110
Phenethylamine	1550	0.022	0.00064	45
Phenpropylamine	610	0.019	0.0016	53
Phenbutylamine	320	0.010	0.0031	100
Phenamylamine	200	0.008	0.0050	120
Phenethylamine	1600	0.022	0.00062	45
Phenethylmethylamine	3800	0.026	0.00026	38
4-Hydroxyphenethylamine	670	0.036	0.0015	28
3,4-Dihydroxyphenethylamine	350	0.044	0.0029	23

$V = V_{max}\,(S)/(K_s + (S))$, or $1/v = K_s/V_{max}(S) + 1/v_{max}$ where

v = velocity

S = substrate concentration

K_s = dissociation constant of enzyme-substrate complex

V_{max} = oxidation rate at infinite substrate concentration.

TABLE XI

Enzyme-Substrate and Enzyme-Inhibitor Constants (Heegaard and Alles, 1943)

Substrate used in variable concentration	Pheniso-propylamine concentration	$\frac{1}{V_{max.}}$	$\frac{1}{K_s}$	$\frac{1}{K_s \quad \frac{K_s}{K_1} \quad I}$	$V_{max.}$	K_s	K_i
	M				micro-liters O_2 per min.	M	M
Amylamine	0.0000	0.023	1650		42	0.00061	
"	0.0020	0.036		600	28		0.0011
"	0.0000	0.023	1650		42	0.00061	
"	0.0010	0.023		780	42		0.0009
Phenethylamine	0.0000	0.025	1360		40	0.00074	
"	0.0200	0.030		950	33		0.047
"	0.0000	0.031	1450		32	0.00069	
"	0.0200	0.037		920	27		0.035
4-Hydroxyphenethylamine	0.0000	0.031	720		32	0.0014	
"	0.0040	0.037		380	27		0.0045
3,4-Dihydroxyphenethyl-amine	0.0000	0.042	510		24	0.0020	
	0.0040	0.054		230	19		0.0033

In Table XI, enzyme-substrate and enzyme-inhibitor constants are shown for a series of amines using the Lineweaver and Burk (1934) equation:

$$\frac{1}{V} = \frac{1}{V_{max}} \cdot \left(K_s + \frac{K_s}{K_i} I\right) \cdot \left(\frac{1}{S}\right) + \frac{1}{V_{max}}$$

The fair agreement between $1/V_{max}$ values for a given substrate with and without inhibitor establishes the competitive nature of the inhibition. However, it should be noted that there are marked variations in K_i values dependent upon the competitive substrate. Thus while phenylisopropylamine is one-third and one-fourth as potent an inhibitor in the presence of tyramine and hydroxytyramine as for amylamine, it is only of the order of one-thirtieth to one-fortieth as active for phenylethylamine inhibitors. A possible explanation for some of these deviations offered by Heegaard and Alles (1943) is that several types of amine oxidases may be present in the preparations used.

Studies of monoamine oxidase distribution (Blaschko, Richter and Schlossmann, 1937; Bhagvat, Blaschko and Richter, 1939; Pugh and Quastel, 1937a) revealed the enzyme to be present in all vertebrates and in many invertebrates. In all animals studied, liver, kidney and intestine showed the highest activity. The following organs were also active: pancreas, heart, lungs, spleen, brain, thyroid, testicle, uterus and adrenal.

The interest in the monoamines of the phenylethylamine type stems from the fact that these compounds have sympathomimetic activity. Physiologically there appears to be a good correlation between deamination of phenylethylamine derivatives, their pressor activity, duration of action, oral efficacy and excretion (Beyer and Morrison, 1945).

DIAMINE OXIDASE

(Histaminase, Diamine dehydrogenase)

Diamine oxidase catalyzes the following general reaction:

$$RCHNH_2 + O_2 \longrightarrow RCH = NH + H_2O_2$$

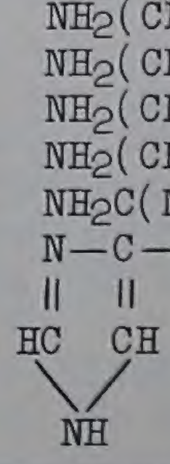
$$RCH = NH + H_2O \longrightarrow RCHO + NH_3$$

The aerobic inactivation of histamine by a thermolabile factor in lung and other tissues was first reported by Best (1929). The name histaminase was given to this enzyme by Best and McHenry (1930). Zeller (1938) reported that kidney preparations of histaminase oxidatively deaminated putrescine, cadaverine and agmatine. Further studies by Zeller (1938a) indicated that one and the same enzyme was involved which led him to give the name diamine oxidase to the system. Similar results were reported by Felix and Zorn (1939) at approximately the same time. While Zeller in particular has been the chief proponent of the identity of histaminase and diamine oxidase, Kapeller-Adler (1944) has questioned this on the basis of comparative oxidation rates with histamine and cadaverine by human serum and placental tissue preparations. More recently, Kapeller-Adler (1946) has reported in a note that partially purified diamine oxidase preparations when dialyzed show a decreased activity with histamine, while the activity with cadaverine, putrescine and agmatine is increased. Interestingly enough, an acetone extract of partially purified enzyme reversed both the effects of dialysis. It thus appears that there may exist a specific histaminase in addition to the diamine oxidase system. A review of the diamine oxidase system has been published by Zeller (1942).

Thirteen different diamines have been shown to be oxidized by the diamine oxidase system (Table XII). The enzyme is widely distributed in many animal species (Zeller, 1942;

TABLE XII

Specificity of Diamine oxidase (Zeller, 1942)

Ethylenediamine	$H_2N(CH_2)_2NH_2$
Trimethylenediamine	$H_2N(CH_2)_3NH_2$
Putrescine	$H_2N(CH_2)_4NH_2$
Cadaverine	$H_2N(CH_2)_5NH_2$
Spermidine	$NH_2(CH_3)_3NH(CH)_4NH_2$
	$NH_2(CH_2)_3NH(CH_2)_3NH_2$
	$NH_2(CH_2)_3NH(CH_2)_5NH_2$
Spermidine homologs	$NH_2(CH_2)_4NH(CH_2)_4NH_2$
	$NH_2(CH_2)_4NH_4(CH_2)_5NH_2$
	$NH_2(CH_2)_5NH(CH_2)_5NH_2$
Spermine	$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$
Agmatine	$NH_2C(NH)NH_2(CH_2)_4NH_2$
Histamine	

Zeller, Birkhauser, Mislin and Wenk, 1939; Zeller, 1941; Zeller and Joel, 1941), bacteria (Werle, 1941; Gale, 1942; Franke and Schillinger, 1944; Roulet and Zeller, 1945) and plants

(Cromwell, 1943). In animals, kidney, intestine and liver have the highest activity. Of interest is the finding that there is a marked increase in diamine oxidase activity of human plasma during pregnancy which falls off abruptly post-partum (Kapeller-Adler, 1944; Zeller, 1940; Ahlmark, 1944; Anrep, Barsoum, and Ibrahim, 1947). This has been associated with a high activity of placental tissue.

Pig kidney has been the chief source of starting material for purification procedures. McHenry and Gavin (1932) early found that partially purified preparations yielded 1 mol. of NH_3 from the terminal amino group of histamine. Stephenson (1943) using partially purified preparations reported indirect evidence for the formation of H_2O_2 during the course of the reaction confirming the findings of Zeller (1940a), and also demonstrated that approximately 1 mol. of aldehyde is formed for each atom of oxygen consumed. Laskowski, Lemley and Keith (1945) succeeded in obtaining preparations of high activity with which one atom of oxygen was utilized and 1 mol. of NH_3 produced for each mol. of histamine oxidized.

Using an enzyme prepared by electrophoretic separation after salt fractionation, Swedin (1943a, 1943b) also found that the uptake of oxygen suddenly decreases when one atom per mol. of histamine has been consumed. Due to the presence of peroxidase activity in most of the preparations previously employed secondary oxidations may occur involving H_2O_2 which will mask the initial deamination reaction.

Zeller, Stern and Wenk (1940) reported that diamine oxidase could be resolved into an apo- and coenzyme, the latter having the properties of a flavine. Swedin (1943a) in his electrophoretic studies observed that the enzyme activity migrated with a flavoprotein. In a later paper (1944) he reported the reversible resolution of the enzyme. The possible role of a flavoprotein in this system is further suggested from the findings by Zeller (1938b) that dialyzed preparations of diamine oxidase can be considerably activated by the addition of a heat-labile, non-dialyzable fraction of rat liver having a high diaphorase activity.

Diamine oxidase is reversibly and competitively inhibited by organic bases such as choline, guanidine and imidazole (Zeller, 1938b, 1941a). Of interest is the observation that thiamine strongly inhibits diamine oxidase, having a higher affinity for the enzyme than histamine (Table XIII). Kidneys from thiamine deficient rats have a higher diamine

TABLE XIII

Michaelis Constants
(Zeller, Schar, and Staehlin, 1939)

	k_M
Putrescine	0.0012
Cadaverine	0.00056
Histamine	0.00050
Thiamine	0.00025

oxidase activity than normal because of this inhibitory effect. All the carbonyl reagents so far tested reversibly inhibit diamine oxidase activity in low concentrations of the order of 10^{-5} M (Zeller, 1938b). Some of the reagents which have been studied are semicarbazide, hydroxylamine, potassium cyanide, phenylhydrazine, etc. As a matter of fact, Zeller (1942) claims that the extraordinary sensitivity of diamine oxidase to low concentrations of the carbonyl reagents can be used to identify the enzyme system. The possibility that an essential carbonyl group is present in diamine oxidase is suggested from these inhibitor studies.

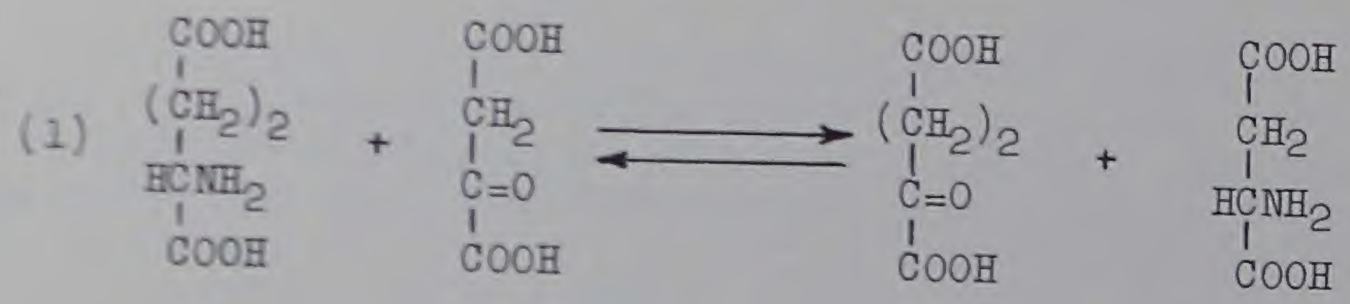
TRANSAMINASES

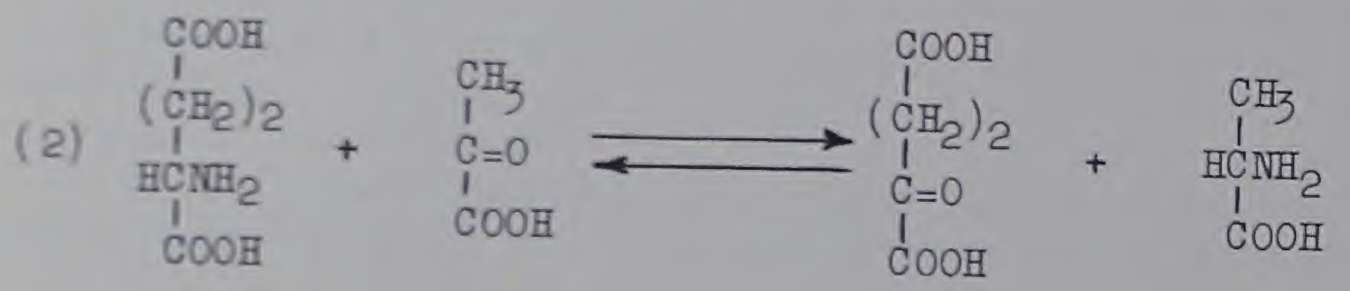
Braunstein and Kritzmann (1937a, b; Kritzmann, 1938a) discovered a reaction involving the non-oxidative transfer of the amino group of an amino acid to an α-ketoacid, the originally discovered reaction involving glutamic acid and pyruvate. They stated the transfer occurred with any α-amino acid to α-ketoglutaric or oxalacetic acids. Later work of

Cohen (1939, 1940a, b) using more refined analytical techniques, limited the reaction to glutamic, aspartic, and cysteic acids, and alanine. A somewhat polemical discussion between the two schools developed in later years.

The original name for the enzymes as suggested by Braunstein was "aminopherases". Cohen proposed (1940a) with some opposition by Braunstein (1947), the term "transaminases". Since either name suggests the function of the enzymes, there is little to choose between them except that transaminase is more consistent with the recent enzymatic nomenclature (transmethylation, transphosphorylation, transimination), and has been employed in reports of the recent critical studies of the enzyme.

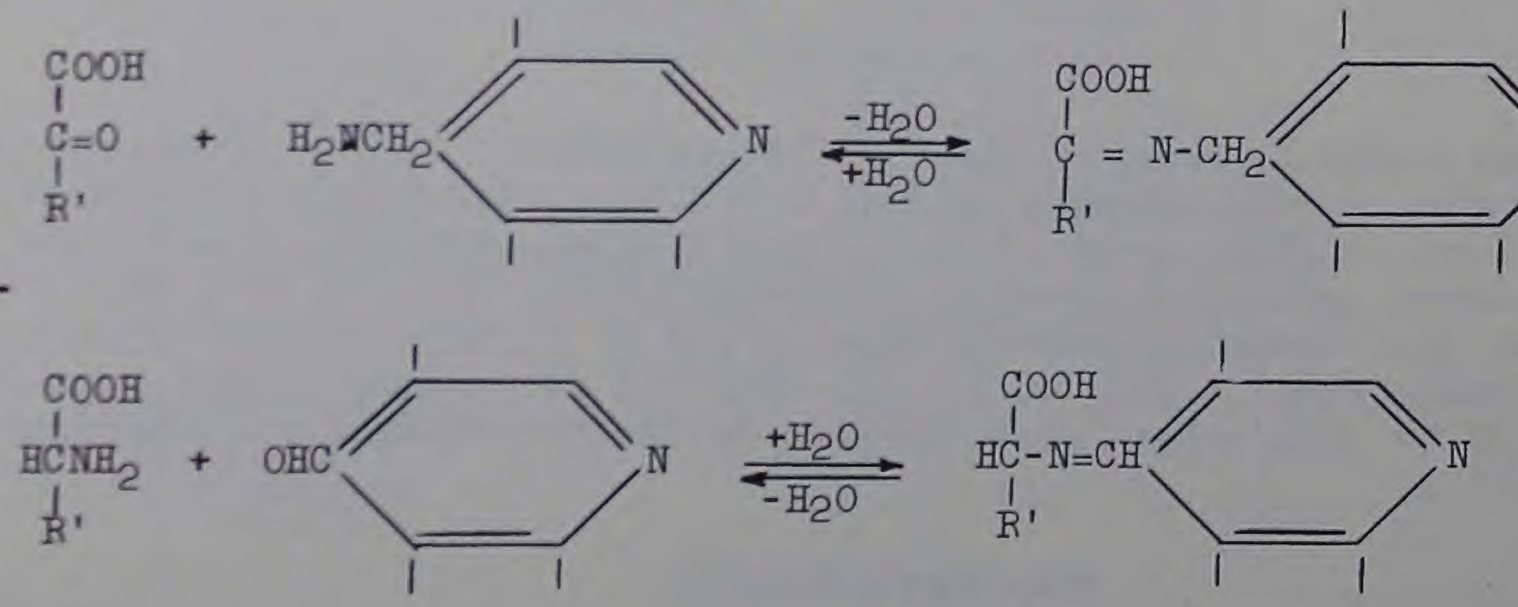
It is now apparent that there are two transaminases (Green, Leloir and Nocito, 1945; O'Kane and Gunsalus, 1947a, b) catalyzing the reactions:

(1) 

(2) 

The enzymes will be referred to in this discussion as the L-glutamic-oxalacetic and L-glutamic-pyruvic transaminases, respectively. The equilibrium constants for the two reactions are circa 3.5 and 1 respectively (Cohen, 1940b, Green, Leloir and Nocito, 1945). In reaction (1), slight activity is observed if oxalacetic acid is replaced by mesoxalic acid, or β-sulfopyruvic acid. In reaction (2), activity can be demonstrated upon replacement of pyruvic acid with α-ketobutyric and mesoxalic acids, but not by phenylpyruvic acid. In the reverse reaction, alanine is not replaceable by N-methyl alanine or D-alanine. L-Glutamic acid is not replaceable (Green, Leloir and Nocito, 1945).

The elucidation of pyridoxal phosphate as the coenzyme of the glutamic-oxalacetic transaminase (Lichstein, Gunsalus and Umbreit, 1945) shortly followed the amazing intellectual tour de force of Snell, who predicted the function from a consideration of the interconversion of pyridoxal and pyridoxamine (Schlenk and Snell, 1945; Snell, 1944, 1945). Schlenk and Fisher (1947) visualize the mechanism as follows:

(3) - 

In like manner, the aldehyde form of the coenzyme reacts with an amino acid to regenerate the original pyridoxamine form, and produces the corresponding keto acid of the second amino acid.

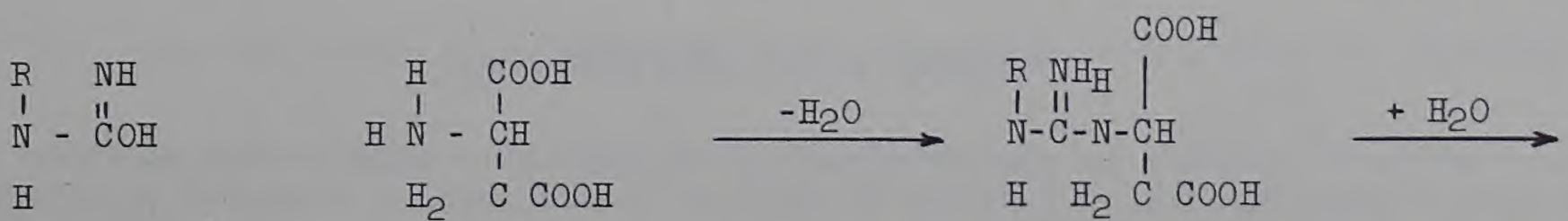
Both holoenzymes have been obtained in purified form by Green, Leloir and Nocito (1945), who employed ammonium sulfate fractionation of an aqueous extract of pig heart, followed by electrophoretic separation. The glutamic-oxalacetic transaminase has two electrophoretic components, both active, and one component in the ultracentrifuge (1.5% yield). The molecular weight is 60,000, and the turnover number 640. The glutamic-pyruvic transaminase was obtained (3.8% yield) as a single electrophoretic and ultracentrifugal component. With an assumed molecular weight of 180,000 (basis for the assumption not stated), the turnover number would be 8600. The high order of activity of the reactions in mammalian tissues can be seen from the estimate, based on the above data, that the two transaminases constitute 1.6 and 0.12%, respectively, of the dry weight of pig heart.

O'Kane and Gunsalus (1947a) obtained the glutamic-oxalacetic apoenzyme in greater than 4.1% yield, and found the dissociation constant of the coenzyme-apoenzyme to be 1.5×10^{-6} (one-half maximum activity at 0.75 micrograms of pyridoxal phosphate per ml.). These authors (1947b) also showed that Braunstein's "aspartic-alanine aminopherase" is an artifact resulting from the combined action of the two transaminases with glutamic acid as a catalyst (the "coenzyme" of "aspartic aminopherase", Braunstein and Kritzmann, 1943).

Transaminases have been demonstrated in many animal, plant, and bacterial cells (Albaum and Cohen, 1943; Cohen and Hekhuis, 1941; Kritzmann, 1938b; Leonard and Burris, 1947; Lichstein and Cohen, 1943). The mammalian enzymes have maximum activity at pH 7.5 (contrasted to 8.6 in oat seedlings) (Albaum and Cohen, 1943), and a K_s (glutamic-oxalacetic enzyme, with both substrates in equal concentration) of 0.014 M (Cohen, 1940b), and are SH sensitive.

TRANSIMINASE

Borsook and Dubnoff (1941) reported the conversion of citrulline to arginine by a transimination reaction involving glutamic acid and related compounds. The reaction was reported to be limited to kidney slices. Cohen and Hayano (1946) later showed that, aerobically, liver homogenates fortified with A.T.P., cytochrome C and magnesium ions in the presence of citrulline and glutamic acid were highly active in catalyzing the synthesis of arginine. Later studies by Cohen and Hayano (1948) indicated that at least two enzymatic steps were involved, one, a soluble enzyme concerned with the synthesis, and a second insoluble enzyme concerned with hydrogen transport and which includes the cytochrome system. However, Ratner (1947) reported that an extract of acetone dried beef liver catalyzes the anaerobic formation of arginine from citrulline and aspartic acid. Aspartic acid, it is claimed, is specific for the reaction. Magnesium ions, phosphoglyceric acid and catalytic amounts of A.T.P. are necessary additions. In addition to arginine, a molar equivalent of malic is formed. The reaction is pictured as follows:



citrulline aspartic acid



arginine malic acid

It is suggested that the high energy phosphate role is that of phosphorylating the hydroxyl group of the isomeric form of citrulline, in which case H_3PO_4 rather than H_2O would be removed in the condensation.

The activity of glutamic acid in the experiments of Cohen and Hayano (1946) is due to transamination to aspartic acid according to Ratner (1947). However, experiments designed to evaluate the respective roles of glutamic and aspartic acids in homogenates indicate that while aspartic acid may be as active as glutamic acid, the latter is highly active under conditions which would preclude transamination to aspartic acid (Cohen and Grisolia, 1948, unpublished experiments). It thus seems possible that there are at least two transiminases, one active with glutamic acid and one with aspartic acid.

Fährlander et al. (1947) have studied the relationship of labile phosphorus to urea synthesis with liver homogenates. Malonic acid inhibition of urea synthesis is associated with a marked decrease in labile phosphorus. This inhibition can be overcome by the addition of fumarate or malate, under which conditions the level of labile phosphate is increased.

TRANSMETHYLASE

Recent investigations indicate that there are at least two types of transmethylase systems (Borsook and Dubnoff, 1947b). One is dependent on oxidative reactions, and the second takes place anaerobically. In the first category is the methylation by methionine of guanidoacetic to creatine and of nicotinamide to N^1 methyl nicotinamide. In the second is the methylation of homocysteine by betaine or choline to form methionine. In homogenates, A.T.P. is necessary for the transmethylase system which methylates guanidoacetic acid in the presence of methionine to form creatine (Borsook and Dubnoff, 1947d). A.T.P. plus a co-metabolite is partially effective in overcoming the anaerobic inhibition of this system. The role of A.T.P. in this reaction is not clear. A new transmethylase using dimethylthetin $[(CH_3)_2S^+CH_2COO^-)]$ as substrate has recently been described by Dubnoff and Borsook (1948). Partially purified preparations from liver and kidney have been studied and found to transfer 1 methyl group from dimethylthetin to homocysteine. In whole liver homogenates, dimethylthetin is 6 times as effective as betaine and 60 times as effective as choline in methionine formation. Dimethylthetin transmethylase has different stability properties from choline and betaine transmethylases.

TRANSULFURASE

Binkley and du Vigneaud (1942) reported that slices and saline extracts of rat liver were capable of converting a mixture of homocysteine and serine to cysteine. The reaction, termed transulfurase*, proceeds anaerobically. The possibility that cystathionine is an intermediate in this reaction is strongly indicated. Of interest in this regard is the finding of Binkley (1944) that cystathionine is cleaved by purified extracts of rat liver when fortified with magnesium or zinc ions and A.T.P. The role of the latter, it is suggested by Binkley, is to provide the energy necessary for cleaving the thio ether by the addition of phosphate at the carbon-sulfur bond.

AMINO ACID DEHYDRASES

This group of enzymes is characterized by an anaerobic deamination reaction resulting from either a desulfhydration (remove of H_2S) as in the case of cysteine or a dehydration as in the case of serine and threonine.

Serine and Threonine Dehydrases

DL serine and DL threonine are anaerobically deaminated by bacteria (Gale and Stephenson, 1938; Chargaff and Sprinson, 1943) and animal tissues (Chargaff and Sprinson, 1943; Binkley, 1943) as follows:

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*transthiolase would appear to be a more appropriate name (Cohen, 1945).

(1) $HOCH_2\ CH(NH_2)\ COOH \xrightarrow{-H_2O} CH_2 = C(NH_2)\ COOH$

$CH_3\ C(=NH)\ COOH \xrightarrow{+H_2O} CH_3COCOOH + NH_3$

(2) $CH_3\ CHOH\ CH(NH_2)\ COOH \xrightarrow{-H_2O} CH_3\ CH = C(NH_2)\ COOH \longrightarrow$

$CH_3CH_2\ C(=NH)\ COOH \xrightarrow{+H_2O} CH_3\ CH_2\ CO\ COOH + NH_3$

Replacement of the hydroxyl hydrogen atom prevents deamination (Chargaff and Sprinson, 1943). Dialyzed mammalian liver extracts are inactive in deaminating DL serine unless divalent cations are added (Chargaff and Sprinson, 1943; Binkley, 1943). A preliminary report by Binkley (1943) suggests that serine dehydrase, cysteine desulfhydrase and enolase may be closely related as judged by mutual inhibition of the substrates.

Cysteine Desulfhydrase

Fromageot, Wookey and Chaix (1939) first described an enzyme in dog livers which produced H_2S from L cysteine which they originally named desulfurase and later renamed desulfhydrase in accordance with the suggestion of Smythe (1944). The desulfhydrase system catalyzes reaction (1) according to Fromageot et al. (1939) and reaction (2) according to Smythe (1942).

(1) $3\ SHCH_2CHNH_2COOH \rightleftharpoons (SCH_2CHNH_2COOH)_2 + H_2S + CH_3CHNH_2COOH$

(2) $SHCH_2CHNH_2COOH \rightleftharpoons CH_3COCOOH + H_2S + NH_3$

Reaction (1) takes place with aqueous extracts of acetone-treated tissue preparations while reaction (2) is observed with more purified preparations and lower cysteine concentrations. Apparently amino acrylic acid is formed as an intermediate in the reaction after removal of H S and may give rise to the endproducts of reactions (1) and (2) in the following manner:

(3) $CH_3C(NH=)COOH + 2\ SHCH_2CHNH_2COOH \longrightarrow (SCH_2CHNH_2COOH)_2 + CH_3CHNH_2COOH$

(4) $CH_3C(NH=)COOH + H_2O \rightleftharpoons CH_3COCOOH + NH_3$

Cystine also forms H_2S with this enzyme (Smythe, 1942). L homocysteine yields small amounts of H_2S and competitively inhibits in the presence of cysteine. Fromageot and Desnuelle (1942) have reported different activity ratios for cysteine and homocysteine and consequently concluded that two independent enzymes may exist. Indirect evidence for the reversal of reaction (2) has been reported by Smythe and Halliday (1942).

In mammals, the enzyme is chiefly found in liver with considerable variation in activity from one species to another. Smythe (1942) gives the following relative activities of livers from different species: rat, 100; dog, 60; human, 50; beef, 18; rabbit, 5; pig, 3; guinea pig, 1. On a similar basis the relative activity of other rat tissues was as follows: kidney, 1; muscle, 1; and brain, 0.

Cysteine desulfhydrase has been reported to be present in Escherichia coli (Desnuelle and Fromageot, 1939) bakers' and brewers' yeast (Binkley, 1943).

Lawrence and Smythe (1943) have partially purified cysteine desulfhydrase from rat liver and have studied a series of inhibitors. Of interest was the finding that cyanide, arsenous oxide and the carbonyl reagents, phenyl hydrazine, semicarbazide, hydroxylanine and sodium bisulfite were strong inhibitors. Amino acids did not inhibit but thiol compounds did, the inhibition by sodium thioglycolate being competitive.

An exocystine desulfhydrase has been found in rat liver by Greenstein and Leuthardt (1944). This enzyme acts on peptides containing cystine in terminal linkage (Leuthardt and Greenstein, 1945). The enzyme is lacking in tumors (Greenstein, 1943).

A recent comprehensive review of sulfur metabolism in animals has been published by Fromageot (1947).

BACTERIAL DEAMINASES

In addition to oxidative deamination, bacteria are capable of deaminating amino acids according to the following reactions:

(1) $R\ CH_2\ CH\ NH_2\ COOH \longrightarrow NH_3 + RCH = CH\ COOH$

(2) $R\ CH\ NH_2COOH + H_2O \longrightarrow NH_3 + RCHOHCOOH$

(3) $R\ CH\ NH_2\ COOH + 2H \longrightarrow NH_3 + RCH_2\ COOH$

(4) $R\ CH\ NH_2\ COOH + R_1\ CH\ NH_2\ COOH + H_2O \longrightarrow 2\ NH_3 + R\ CO\ COOH + R_1\ CH_2\ COOH$

These enzymatic reactions and the factors influencing them are reviewed by Gale (1940, 1943).

AMIDE BOND SYNTHESIS (SYNTHETASES)

The increasing interest in the enzymatic synthesis of amide bonds stems from the fact that our knowledge of the mechanism of peptide-bond synthesis is all too inadequate. In view of the fact that the synthesis of amide bonds in general represents endergonic reactions which involve the same order of free energy of formation, viz. 3000 calories, any information that can be gained from systems which catalyze the formation of amide-bonds can be expected to aid in the elucidation of the mechanism of peptide-bond synthesis. Four systems dealing with amide-bond synthesis have been studied with cell-free preparations:

(1) acetylation of aromatic amines
(2) glutamine synthesis
(3) hippuric and p-aminohippuric acid synthesis
(4) citrulline synthesis

These reactions have in common the requirement for A.T.P. While the specific role of A.T.P. is not clear in these reactions, it is highly probable that it is functioning to form a high energy phosphate derivative of one or more of the substances concerned, or of a substrate-coenzyme complex. Since the overall reactions are complex as regards the number of enzymatic steps in the systems used, they will be discussed as overall reactions unless more detailed information is available.

Acetylation of aromatic amines

Lipmann (1945) first succeeded in demonstrating acetylation of aromatic amines by liver homogenates and extracts of acetone-dried liver preparations. Pigeon liver was

particularly active. The reaction takes place anaerobically if A.T.P. is added. Acetate was found to be twice as active as acetoacetate or pyruvate. However, acetyl phosphate does not appear to be an intermediate since it is not active with this system. Of the aromatic amines, sulfanilamide was most actively acetylated followed by p-aminobenzoic acid, sulfthiazole and sulfadiazine. The enzyme system, which is soluble, is inactivated by dialysis, but reactivation results when boiled liver juice is added. The active coenzyme, called coenzyme A, for this system was later shown by Lipmann et al. (1947) to contain pantothenic acid. Of interest is the finding that the same coenzyme is required for the synthesis of the ester linkage in acetyl choline by brain extracts (Lipmann and Kaplan, 1946).

Glutamine synthesis

Speck (1947) reported the synthesis of glutamine from glutamic acid and ammonia in extracts of acetone-dried pigeon liver. A.T.P. and magnesium ions are required and the reaction proceeds anaerobically. Cysteine accelerates the rate of the reaction. Inorganic phosphate is liberated from A.T.P. at a rate paralleling glutamine synthesis. Hydroxylamine may be substituted for ammonia and the product of the reaction can then be measured as hydroxamic acid by the method of Lipmann and Tuttle (1945). The reaction is inhibited by 10^{-3} M sodium fluoride.

Two independent studies conducted simultaneously with that of Speck were reported by Bujard and Leuthardt (1947) and Leuthardt and Bujard (1947) and Elliott (1948). The former investigators demonstrated the synthesis of glutamine in liver homogenates of rat and guinea pig liver from glutamic acid and ammonia and showed the dependence of the reaction on A.T.P. and magnesium ions. Elliott reported the synthesis of glutamine using extracts of acetone-dried gray matter of sheep's brain and also showed the requirement for A.T.P. and magnesium ions. Further, Elliott showed, using an enzyme preparation free of ATPase, that in the presence of hydroxylamine the formation of hydroxamic acid is equivalent to the liberation of inorganic phosphate from A.T.P. The glutamine synthetase was also demonstrated in cell-free extracts of Staphylococcus-Aureus. Further details of these studies were reported by Elliott (1948a, b).

Hippuric and p-aminohippuric acid synthesis

Liver and kidney contain an enzyme system which is capable of synthesizing hippuric acid from benzoic acid and glycine (Borsook and Dubnoff, 1940) and p-aminohippuric acid from p-aminobenzoic acid and glycine (Cohen and McGilvery, 1946). The synthesis of both compounds takes place with the same enzyme system since benzoic acid competitively inhibits the synthesis of p-aminohippuric acid (Unpublished studies, Cohen and McGilvery, 1947). The synthesis of p-amino hippuric acid by rat liver homogenates was first reported by Cohen and McGilvery (1947a) who showed that in the presence of A.T.P. an appreciable anaerobic synthesis could be realized. At about the same time, Borsook and Dubnoff (1947a) reported that the addition of adenylic acid to guinea pig liver homogenates considerably enhanced the aerobic synthesis of hippuric acid. A detailed study of p-aminohippuric synthesis by rat liver homogenates (Cohen and McGilvery, 1947) revealed the optimum conditions for this system. Thus the formation of p-aminohippuric acid was stimulated by the addition of potassium and magnesium ions, cytochrome C, and metabolites of the citric acid cycle. Of considerable interest was the finding that fumarate at 2.5×10^{-3} M concentration eliminated a marked dilution effect in addition to maintaining the optimum rate of the reaction for a longer time. Further studies by Cohen and McGilvery (1947b) showed that the enzyme system forming p-aminohippuric acid (and hippuric acid) was exclusively associated with the large insoluble particles of the rat liver and kidney cell. The enzyme preparation is unusually sensitive to hypo- or hypertonic salt solutions, freezing and acetone drying. Adenylic acid cannot replace A.T.P. in the absence of an oxidizable substrate. The enzyme system is highly specific for hippuric or p-aminohippuric acid synthesis since glycine cannot be replaced by any one of a large series of amino acids, and peptides investigated (Cohen and McGilvery, unpublished studies). Of interest in this connection was the finding that N-phospho-glycine was not significantly active in this system. Hippuric acid synthetase would appear to be the most descriptive name for this system.

Citrulline synthesis

The synthesis of citrulline from ornithine by liver homogenates was first reported by Cohen and Hayano (1947a). The optimum conditions for the homogenate system included the presence of glutamic acid, A.T.P. or adenylic acid and inorganic phosphate ions in addition to ornithine, ammonia and carbon dioxide (Cohen and Hayano, 1947b). Upon centrifugation of rat liver homogenates the total citrulline synthesis activity remains in the insoluble residue (Cohen and Hayano, 1948).

The potent citrulline synthetase system in the insoluble residue requires magnesium ions for optimum activity. The system is unstable to freezing or drying. Of considerable interest is the finding that caffeine and theophylline are rather potent inhibitors which may explain the inhibitory effects of these purines on urea synthesis in liver slices and in man in vivo (Bernheim and Bernheim, 1945). Evidence has been presented by Cohen and Grisolia (1948) that the role of glutamic acid is that of acting as the primary carbon dioxide acceptor. It was found that carbamyl glutamic acid was 10-15 times as active in citrulline synthesis as glutamic acid when a carbon dioxide free medium was employed. A study of ornithine-catalyzed urea formation in guinea pig liver homogenates has been reported by Borsook and Dubnoff (1947c).

AMINO ACID DECARBOXYLASES

DECARBOXYLASES OF BACTERIAL ORIGIN

The amino acid decarboxylases catalyze the following general reaction:

$$R\ CH(NH_2)COOH \longrightarrow R\ CH_2NH_2 + CO_2$$

An excellent review of this subject has been published by Gale (1946). Of a large number of amino acids and bacterial strains investigated by Gale and others, only the following six amino acids have been shown to be capable of decarboxylation by bacterial enzymes: lysine, ornithine, arginine, tyrosine (and 3:4 dihydroxyphenylalanine), histidine and glutamic acid. This has led Gale to make the generalization that in order for an amino acid to evoke the formation of a decarboxylase in bacteria, it must possess: (1) a free-carboxyl group in the carbon-1 position; (2) a free amino group in the carbon-2 position; (3) a free terminal polar group; and (4) the natural l-configuration. However, the decarboxylation of L-phenylalanine by Streptococcus faecalis R preparations has recently been demonstrated by McGilvery and Cohen (1948).

The presence of amino acid decarboxylases in bacteria is determined by a number of factors, the following of which are most important according to Gale (1946):

1. The organism concerned must possess such enzymes in its potential enzymic constitution.
2. Growth must take place in the presence of the specific substrate.
3. The organisms must be capable of synthesizing codecarboxylase, or the growth medium must contain certain factors involved in the formation of codecarboxylase.
4. The growth medium must be acid.
5. With some organisms, amino acid decarboxylases are formed to a significant extent only if growth occurs at temperatures lower than 30° C.
6. The enzymes are fully developed within the organism only at the end of active cell division.

The preparation and properties of the specific amino acid decarboxylases available for analytical purposes are shown in Table XIV.

TABLE XIV

Specific Amino-Acid Decarboxylase Preparations for Estimation of Amino-Acids (Gale, 1947)

Enzyme	Organism (N.C.T.C. no.)	Conditions of culture: Medium	Conditions of culture: Temp.	Time (hr.)	Specific preparation	Activity Maintained	Manometric estimation: Buffer	Manometric estimation: CO_2 evolution as % theory
L-Lysine decarboxylase	Bact. cadaveris (6578)	3% casein digest 2% glucose (1l. medium = 50 estimations)	25°	24	Acetone (5 vol.)-ether-dried powder, kept 3 days at 0° before use Use ca. 10 mg. per test	4-6 weeks in desiccator	m/5 phosphate, pH 6.0 + acid-tip	92 98
L-Arginine decarboxylase	Esch. coli (7020)	3% casein digest 2% glucose (1l. medium = 40 estimations)	25°	24	Acetone (5 vol.)-ether-dried powder Use ca. 10 mg. per test	4-6 weeks in desiccator	m/5 phosphate-citrate, pH 5.2	95
L-Histidine decarboxylase	Cl. welchii, B.W. 21 (6785)	3% casein digest 2% glucose Heart muscle (1l. medium = 20 estimations)	37°	16	Acetone (3 vol. for 30 min.)-ether-dried powder Use ca. 30 mg. per test	2-3 months in desiccator	m/5 acetate, pH 4.5	96
L-Ornithine decarboxylase	Cl. septicum, PIII (547)	3% casein digest 2% glucose Heart muscle (1l. medium = 20 estimations)	37°	16	Washed suspension of organism 20-30 mg. dry weight cells/ml. Use 0.5 ml. per test	24 hr.	m/5 phosphate-citrate, pH 5.5 + acid-tip	92 98
L-Tyrosine decarboxylase	S. faecalis (6783)	3% casein digest 1% glucose 0.1% marmite (1l. medium = 50 estimations)	37°	16	Acetone (5 vol.)-ether-dried powder Use ca. 10 mg. per test	2-6 weeks in desiccator	m/5 phosphate-citrate, pH 5.5	96
L-Glutamic acid decarboxylase	Cl. welchii, S.R. 12 (6784)	3% casein digest 2% glucose Heart muscle Hydrogen (1l. medium = 30 estimations)	37°	12	Washed suspension of organism 20-30 mg. dry weight cells/ml. Use 0.5 ml. per test	48 hrs. in H_2	m/5 acetate, pH 4.5	98

Bacterial decarboxylases require pyridoxal phosphate as coenzyme (Umbreit and Gunsalus, 1945; Baddiley and Gale, 1945).

Of interest is the fact that this is the same coenzyme functioning in the transaminase systems (Lichstein, Gunsalus and Umbreit, 1945) and in the tryptophanase system of E. coli (Wood, Gunsalus and Umbreit, 1947).

Partial purification and resolution of apoenzyme and codecarboxylase has been effected for the following decarboxylase systems: lysine, Gale and Epps (1944); tyrosine, Epps (1944) and Umbreit, Bellamy and Gunsalus (1945); arginine and glutamic acid, Taylor and Gale (1945) and Umbreit and Gunsalus (1945); ornithine, Taylor and Gale (1945). The unsuccessful attempts to dissociate the histidine decarboxylase system by Epps (1945) has led to the suggestion that this enzyme is devoid of codecarboxylase.

An interesting observation on the effect of detergents on glutamic acid and histidine decarboxylases from Clostridium welchii organisms is reported by Krebs (1948). The decarboxylation of glutamic acid and glutamine (which is converted to ammonium glutamate by the enzyme preparation previous to decarboxylation) is markedly accelerated by the presence of 0.02 per cent cetyl trimethyl ammonium bromide a cationic detergent. An anionic detergent, sodium dodecyl sulfate inhibited the decarboxylation of glutamic acid. On the other hand, histidine decarboxylation was inhibited by cetyl trimethyl ammonium bromide while sodium dodecyl sulfate was without effect.

Of interest is the finding that the glutaminase activity of this organism is of high specificity and activity making it suitable for glutamine determination.

DECARBOXYLASES OF ANIMAL ORIGIN

The animal amino acid decarboxylases have not been investigated with as much success as the bacterial enzymes. Aside from dopa (dihydroxyphenylalanine) decarboxylase, the enzyme preparations used have had low activity and in many instances the characterization of the endproduct amine has involved biological rather than chemical methods. The subject has been reviewed recently by Blaschko (1945). The following amino acids have been reported to be decarboxylated by animal tissues: histidine, tryptophane, tyrosine, dopa, and cysteic acid. Activity is chiefly confined to kidney and liver. The role of a cofactor has not been established for these enzyme systems. However, Green, Nocito and Leloir (1945) have reported in a footnote that they have succeeded in partially purifying dopa decarboxylase by ammonium sulfate fractionation. Dialysis against 0.002 M ammonia at 0° for four days resulted in almost complete inactivation. Reactivation required pyridoxal phosphate in addition to cysteine or glutathione. Of interest is the finding that the animal amino acid decarboxylases are strongly inhibited by the ketone reagents, such as hydrazine, hydroxylamine, semicarbazide, etc. The bacterial and plant amino acid decarboxylases are similarly inhibited indicating that both systems have a carbonyl group in common, in all probability the aldehyde group of pyridoxal.

TABLE XV

Comparison of Animal and Bacterial Decarboxylases on Different Hydroxyphenylalanine Compounds. (Blaschko and Stanley, 1948)

Enzyme source	Substrates			
	Tyrosine	meta tyrosine	3:4 dihydroxyl phenylalanine	2:5 dihydroxy phenylalanine
Guinea pig kidney	-	+	+	+
Strep. faecalis	+	-	+	-

While bacterial tyrosine decarboxylase (from Strep. faecalis) also reacts with 3:4 dihydroxyphenylalanine, animal dopa decarboxylase does not decarboxylate tyrosine. Recent studies by Blaschko and Stanley (1948) indicate that the mammalian enzyme requires the presence of an hydroxyl group in the meta position while the bacterial enzyme requires it in the para position. Thus both meta-tyrosine and 2:5 dihydroxyphenylalanine are decarboxylated by animal dopa decarboxylase but not by bacterial enzyme preparations. Of interest is the observation that meta-tyrosine is oxidized by ophio-oxidase of cobra venom and pig kidney d-amino acid oxidase. A comparison of animal and bacterial decarboxylase activity on different hydroxyphenylalanine compounds is shown in Table XV.

DECARBOXYLASES OF PLANT ORIGIN

The decarboxylation of glutamic acid by higher plants was studied by Schales, Mims and Schales (1946) and Schales and Schales (1946a). Of a large series of plants investigated, squash, avacado and green pepper were found to be particularly active. Carrots provided a convenient source for study of the system. Carrot extracts become inactive when dialyzed but can be reactivated by the addition of pyridoxal phosphate. Thus the same prosthetic group appears to be functioning in both the bacterial and plant and possibly the animal decarboxylases.

MISCELLANEOUS

Histidase

Histidase is a hydrolytic enzyme which catalyzes the rupture of the imidazole ring to give rise to glutamic acid, ammonia and formic acid. The enzyme is found only in vertebrate liver. A recent investigation on purified histidase has been reported by Walker and Schmidt (1944) and includes a complete bibliography of previous studies. Walker and Schmidt present evidence for the formation of α-formamidino glutamic acid as an intermediate in the histidase reaction.

Arginase

Arginase catalyzes the hydrolysis of arginine to ornithine plus urea. Its role in the urea cycle of Krebs and Henseleit (1932) is well known. Recent studies on purification include those of Richards and Hellerman (1940). Hunter and Downs (1944), Mohamed and Greenberg (1945), Greenberg and Mohamed (1945) and Bach (1946). Bach obtained crystalline material of high activity. Of interest was his finding that the purest fractions did not require activation by divalent cations.

Glutaminase

Glutaminase catalyzes the hydrolysis of the amide, glutamine. More recent studies following the original investigation of Krebs (1935a) are those of Archibald (1944) and Krebs (1948). Krebs (1948) has found that washed cultures of Clostridium welchii, strain SR12 have a highly specific glutaminase which is active at pH 4.9.

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Chapter XII

ENERGY RELATIONS IN METABOLIC REACTIONS

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INTRODUCTION

It is only in relatively recent years that biochemists have fully realized the necessity for coupling mechanisms between the exergonic* reactions of carbohydrate metabolism and the endergonic reactions associated with synthesis of cell substance, muscle contraction, osmotic work, and the like. The classical biochemical attitude was somewhat analogous to that of the man who tried to elucidate the functioning of an automobile solely by a study of the end products of gasoline combustion and the mechanism of their formation. The equation for muscle glycolysis, for instance, was written

$$\text{Glycogen} \longrightarrow 2 \text{ lactic acid}$$

Much effort was expended in attempts to elucidate the intermediate steps in this postulated reaction. No cognizance was taken of the indisputable fact that if the over-all reaction proceeded according to the above equation, none of the free energy of reaction could be available for muscle contraction. Even after it was known that phosphocreatine synthesis accompanied glycolysis in muscle, the glycolysis reaction was still represented by the above equation. Investigators still made no specific attempts to determine the nature of the energy coupling mechanism. This mechanism was discovered accidentally, and even then its importance was not at first recognized.

At present, much effort is being devoted to investigation of metabolic energy coupling mechanisms. A discussion of the basic nature of these mechanisms, and of the factors involved in energy metabolism, is therefore appropriate. The material to be presented will deal primarily with coupling mechanisms, but energy conversion (e.g., utilization of chemical energy for mechanical or osmotic work), factors governing energy efficiency, certain control mechanisms, and other related topics will receive attention.

FUNDAMENTAL RELATIONSHIPS

Before discussing energy transfer in biological systems, we will review briefly a few of the basic concepts of energy change in chemical reactions. In the reaction

$$A + B \rightleftharpoons C + D$$

$$K = \frac{(C)\ (D)}{(A)\ (B)}$$

where K is the equilibrium constant and the concentrations of the reactants are those at equilibrium. The change in the free energy content of the system (ΔF) occurring when the reaction takes place is always negative if the reaction proceeds spontaneously from left to right; that is, any chemical reaction whatever, occurring at a measurable velocity, results in a decrease in the free energy content of the universe. Quantitatively, the free energy change in the standard state, when all reactants are present at unit concentration**, is

$$-\Delta F^{\circ} = RT \ln K$$

---

*The term "exergonic" is descriptive of a reaction which proceeds spontaneously from left to right as written, and the term "endergonic" is descriptive of a reaction that proceeds spontaneously in the reverse direction, or from right to left. Hydrolysis of sucrose in aqueous solution is exergonic; decomposition of water into hydrogen and oxygen is highly endergonic.

** Unit concentration may be considered to be molal for dissolved solids or liquids, one atmosphere for gases, and unity for water in dilute aqueous solutions. It should be noted that a liquid in equilibrium with a gas at one atmosphere can be considered to have dissolved in it unit concentration of the gas, since at equilibrium the solution reaction has zero free energy change. For accuracy, activities rather than concentrations should be considered, but because most biological reactant concentrations are low, the use of concentrations will not introduce a large error.

or, at 25°C.,

$$-\triangle F^{o} = 1370 \log_{10} K \text{ calories per mole.}$$

At any concentration of reactants

$$\triangle F = \triangle F^{o} + RT \ln \frac{(C)\ (D)}{(A)\ (B)}$$

or, at 25°C.,

$$\triangle F = \triangle F^{o} + 1370 \log_{10} \frac{(C)\ (D)}{(A)\ (B)} \text{ calories per mole}$$

or

$$-\triangle F = 1370 \left[\log_{10} K - \log_{10} \frac{(C)\ (D)}{(A)\ (B)}\right]$$

It will be noted that at equilibrium reactant concentration, the two terms of the above expression are equal and of opposite sign; hence, at equilibrium, where the reaction velocity reaches zero, the free energy change is also zero. It is also obvious that in reactions proceeding under conditions remote from those of equilibrium, the decrease in free energy brought about by the reaction is relatively large.

A few examples will illustrate the points brought out above.

(1) How completely will hydrogen be oxidized by atmospheric oxygen? The standard free energy ($-\triangle F^{o}$) for the reaction between hydrogen and oxygen to produce water is 56,560 calories per mole of water.

$$2\ H_2 + O_2 = 2H_2O$$

At equilibrium at 25°

$$1370 \log \frac{(H_2O)^2}{(H_2)^2\ (O_2)} = 2 \times 56{,}560$$

At equilibrium in aqueous solution, in the presence of 0.2 atmospheres of O_2

$$1370 \log \frac{(1)^2}{(H_2)^2 (0.2)} = 113{,}120$$

$$(H_2) = 1.1 \times 10^{-41} \text{ atmospheres.}$$

In this very exergonic reaction, the hydrogen pressure at equilibrium would be so low that the reverse reaction could not be demonstrated experimentally.

(2) If catalytic hydrolysis of 0.1 M ethyl acetate is 99% complete in dilute aqueous solution, what is $\triangle F^{o}$? Since the water concentration remains at unity during the reaction, this reactant can be neglected, and

$$-\triangle F^{o} = 1370 \log K$$

$$-\triangle F^{o} = 1370 \log \frac{(AcOH)\ (EtOH)}{(EtOAc)}$$

or, approximately,

$$-\Delta F^{o} = 1370 \log \frac{(0.099)\ (0.099)}{(0.001)}$$

$$-\Delta F^{o} = 1356 \text{ calories}$$

ENERGY COUPLING IN CONSECUTIVE REACTIONS

There is only one mechanism for transfer of chemical bond energy directly from one chemical reaction to another. The two reactions must have a common reactant. What is meant by energy transfer in the above sense is the utilization of a part of the free energy of an exergonic reaction to "drive" a normally endergonic reaction. Consider two reactions

(I) $A \rightleftharpoons B$ ($K_1 = 0.01$; $\Delta F^{o} = +2470$ cal.)

(II) $B \rightleftharpoons C$ ($K_2 = 1000$; $\Delta F^{o} = -4110$ cal.)

Reaction (I) is endergonic, and will proceed in the reverse direction (from right to left) unless the concentration of B is less than 1% of the concentration of A. Reaction (II) is exergonic, and will proceed toward the right until the concentration of B is reduced to 0.1% of the concentration of C. If these two reactions proceed consecutively, it may be seen qualitatively that reaction (II) will tend to get rid of B as fast as it is formed by reaction (I), and if B is held at a concentration sufficiently low, reaction (I) will proceed in a forward direction. The quantitative relation will become apparent if we consider the over-all reaction $A \rightleftharpoons C$.

$$K = K_1 K_2 = 10; \quad \Delta F^{o} = (2470 - 4110) = -1370 \text{ cal.}$$

Thus, the over-all reaction will proceed until the concentration of C is ten times that of A. The net result is that, effectively, the free energy of reaction (II) has been transferred to reaction (I), enabling it to proceed in a forward direction.

This type of energy coupling between chemical reactions is the only one known to occur in biological systems. It cannot be overemphasized that endergonic reactions, by definition, do not occur in biological systems or anywhere else. A reaction which is endergonic under usual concentration conditions may become exergonic if subsequent reactions remove one or more of the products sufficiently completely. When the concentration of a compound is known, however, many reactions which might be postulated are automatically ruled out. For example, the sucrose concentration in sugar cane juice is more than 0.5 M. If the plant synthesizes sucrose by the reaction: glucose + fructose = sucrose + water, we may reason that since ΔF^{o} for this reaction is probably in the neighborhood of +6000 calories, fantastically high monosaccharide concentrations and very low water concentrations would be necessary to cause the synthesis to proceed. Hence, it becomes extremely likely that this mechanism of synthesis is not the one used by the plant.

PHOSPHORUS COMPOUNDS AS COMMON REACTANTS

In order for an exergonic reaction to drive a normally endergonic one, it is necessary that the two reactions be consecutive, (the driven reaction occurring first) and that the two reactions have a common reactant. These requirements are sufficiently stringent that one might expect to encounter difficulties in explaining such phenomena as the utilization of the energy of oxidation of some single metabolite for driving the many endergonic reactions involved in growth. One of the most elegant of the many ingenious mechanisms occurring in metabolism is the utilization of a single common reactant in a large number of reactions. This common reactant is adenosine triphosphate or sometimes another

high-energy phosphorus compound. Its synthesis from inorganic phosphate is endergonic at biological reactant concentrations. The general mechanism of synthesis of high-energy phosphate compounds may be expressed as follows:

(I) Hypothetical driving reaction: $AOH \rightleftharpoons BOH$ (exergonic, ΔF^{o} negative)

(II) Hypothetical driven reaction: $ROH + H_3PO_4 \rightleftharpoons RH_2PO_4 + H_2O$ (endergonic, ΔF^{o} positive)

Actual mechanism:

(III) $AOH + H_3PO_4 \rightleftharpoons AH_2PO_4 + H_2O$ (endergonic)

(IV) $AH_2PO_4 \rightleftharpoons BH_2PO_4$ (exergonic)

(V) $BH_2PO_4 + ROH \rightleftharpoons BOH + RH_2PO_4$ (slightly exergonic)

Reaction (IV) is identical with the postulated driving reaction (I) except that the phosphorylated form of the metabolite undergoes the reaction. Reaction (IV) maintains the concentration of AH_2PO_4 at a level sufficiently low to render reaction (III) exergonic, i.e., to "drive" it. Since the sum of equations (III), (IV), and (V) is the same as the sum of equations (I) and (II), an observer able to analyze only for original reactants and final products might assume that reactions (I) and (II) actually occurred, and that energy coupling occurred by some occult means. A more critical observer would reason that reaction (II), being endergonic, could not possibly occur as written, and that reaction (I) could not possibly occur as written if it were to drive reaction (II), since no common reactant is present. (An interesting related fact is that biochemists spent three decades trying to explain why yeast extracts refused to carry out alcoholic fermentation without "abnormal" accumulation of phosphate esters. Not once during this period was the necessity for reactants common to energy-yielding and energy-consuming reactions recognized.)

A high-energy phosphate compound having a standard free energy of hydrolysis of -10,000 to -15,000 calories per mole may be used in a number of ways to drive reactions which are normally endergonic. Water may be removed in the synthesis of polysaccharides, amides, and esters by the following general mechanism:

Hypothetical driving reaction:

$$RH_2PO_4 + H_2O \rightleftharpoons H_3PO_4 + ROH \text{ (exergonic)}$$

Hypothetical driven reaction:

$$R_1OH + R_2OH \rightleftharpoons R_1OR_2 + H_2O \text{ (endergonic)}$$

Actual mechanism:

$$RH_2PO_4 + R_1OH \rightleftharpoons ROH + R_1H_2PO_4 \text{ (exergonic)}$$

$$R_1H_2PO_4 + R_2OH \rightleftharpoons H_3PO_4 + R_1OR_2$$

As in the previous example, neither the driving reaction nor the driven reaction actually occurs as written.

Further discussion of utilization of phosphate bond energy will be given later, but the preceding will illustrate the point that high-energy phosphorus compounds may serve as generally useful common reactants in energy coupling. As far as is known at present, these compounds constitute the general mechanism by means of which energy from metabolite oxidation or fermentation is made available for growth reactions.

BIOLOGICAL SYNTHESIS OF HIGH-ENERGY PHOSPHORUS COMPOUNDS

The energetic aspects of biological synthesis of high-energy phosphates have been discussed by Kalckar (1, 2), Lipmann (3, 4) and, recently, by Ogston and Smithies (5). It is sufficient for the present discussion to point out that while the free energy of hydrolysis of ordinary phosphate esters or amides is only a few thousand calories, the hydrolysis energy of a phosphorylated resonant structure such as a carboxyl group, an enolic hydroxyl, or a guanidine group, is of the order of 10,000 to 12,000 calories in neutral solution.

The first system in which the detailed mechanism of phosphorylation was elucidated (6, 7) was the phosphorylation accompanying alcoholic fermentation. The phosphorylation reaction in muscle glycolysis was quickly found to be identical. The reactions may be summarized as follows:

$$\text{1/2 1,6-diphosphofructose} \rightleftharpoons \text{3-phosphoglyceraldehyde} \quad (\Delta F^\circ = +2500 \text{ cal.})$$

$$\text{3-phosphoglyceraldehyde} + H_3PO_4 \rightleftharpoons \text{1,3-diphosphoglyceraldehyde} \quad (\Delta F^\circ = +2000 \text{ cal.})$$

$$\text{1,3-diphosphoglyceraldehyde} + CoI \rightleftharpoons \text{1,3-diphosphoglyceric acid} + CoIH_2 \quad (\Delta F^\circ = 0 \text{ cal.})$$

$$\text{1,3-diphosphoglyceric acid} + ADP \rightleftharpoons ATP + \text{3-phosphoglyceric acid} \quad (\Delta F^\circ = -5000 \text{ cal.})$$

$$CoIH_2 + \text{acetaldehyde} \rightleftharpoons \text{ethyl alcohol} + CoI \quad (\Delta F^\circ = -5500 \text{ cal.})$$

Sum:

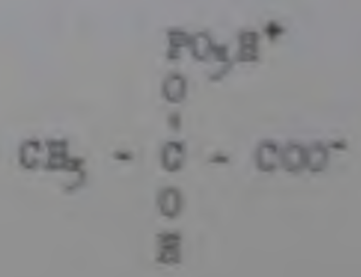
$$\text{1/2 1,6-diphosphofructose} + ADP + H_3PO_4 + \text{acetaldehyde} \rightleftharpoons \text{3-phosphoglyceric acid} + \text{ethyl alcohol} + ATP \quad (\Delta F^\circ = -6000 \text{ cal.})$$

The standard free energy values given are only approximations, but it may be seen that the over-all reaction is strongly exergonic. The bulk of the bond energy of the carboxyl phosphate arises when the aldehyde phosphate (low energy) is oxidized to a carboxyl phosphate (high energy). The free energy of the oxidation of aldehyde to carboxylate ion would be much higher if the free aldehyde group, rather than the aldehyde phosphate, were oxidized. Thus, a large part of the free energy stored in the carboxyl phosphate bond arises from the fact that in the oxidoreduction step, some of the bond energy of the glyceraldehyde is transferred to the phosphate bond rather than being lost. This represents a modification of the simple hypothetical case discussed on page 259.

The only other phosphorylating reaction of which we have detailed knowledge is the phosphorylation accompanying pyruvate dissimilation by various organisms. In this reaction, which has been discussed by Lipmann (4), the exact mechanism is unknown, but the driving reaction is the conversion of pyruvate to acetate, and the high-energy phosphate produced is acetyl phosphate. The over-all reaction, as it occurs in Clostridium butylicum extracts is

$$CH_3COCOOH + H_3PO_4 \rightleftharpoons CH_3COH_2PO_4 + H_2 + CO_2$$

The free energy change in the reaction is small, since practically all of the energy available in the driving reaction is utilized in production of high-energy phosphate. According to Lipmann (4), the mechanism may involve primary addition of phosphate to pyruvate with the formation of



Very little is known concerning the mechanism of the aerobic phosphorylation reactions of animal metabolism. The recent work of Cross, Covo, Taggart, and Green (8) indicates that 14 moles of high-energy phosphate are produced when one mole of pyruvate is oxidized. Ogston and Smithies (5) calculate that $\Delta F^{o} = -274,310$ calories for oxidation of pyruvate by atmospheric oxygen. Correction to physiological values for pyruvate, oxygen, and carbon dioxide concentrations gives approximately -279,000 calories. Reckoning 16,700 calories as equivalent to one phosphorylation, 16 or 17 phosphorylations are possible (at equilibrium, which of course does not occur). A value of 14 is thus reasonable. Some of the implications of the acceptance of such a value will be discussed later. It is sufficient to mention here that each hydrogen pair, during its transport from a metabolite of the tricarboxylic acid cycle to oxygen, must be involved in phosphorylation reactions at two or three different stages. If 14 moles of high-energy phosphate are produced by oxidation of one mole of pyruvate, 30 or more should be produced by oxidation of one mole of glucose. If the pyruvate is produced by the same mechanism as in glycolysis, the number would be 30. If additional phosphorylation accompanies the oxidation of the reduced coenzyme arising from pyruvate production, the number would be greater than 30.

BIOLOGICAL UTILIZATION OF HIGH-ENERGY PHOSPHATES

Since, apparently, the chief mechanism of energy coupling in metabolic systems involves synthesis of high-energy phosphate, it follows that most of the normally endergonic reactions involved in life processes must be made exergonic by mechanisms involving loss of high-energy phosphate. That is, a high energy phosphorus compound must be a reactant common to both the energy-yielding and the energy-consuming reaction. In a few cases, considerable information exists concerning the mechanism of this coupling. The synthesis of sucrose (9) will serve as a model of one type of coupling.

Driven reaction (endergonic):

$$\text{Glucose} + \text{fructose} \rightleftharpoons \text{sucrose} + H_2O \quad (\Delta F^{o} = +6000 \text{ cal.})$$

Driving reaction (exergonic):

$$\text{ATP} \rightleftharpoons \text{ADP} + H_3PO_4 \quad (\Delta F^{o} = -13,000 \text{ cal.})$$

Actual mechanism:

$$\text{ATP} + \text{glucose} \rightleftharpoons \text{glucose-1-phosphate} + \text{ADP} \quad (\Delta F^{o} = -8800 \text{ cal.})$$

$$\text{Glucose-1-phosphate} + \text{fructose} \rightleftharpoons \text{sucrose} + H_3PO_4 \quad (\Delta F^{o} = +1800)$$

The free energy values are only estimates, because of the absence of data on the energy of hydrolysis of sucrose. It will be seen, however, that energy transfer is accomplished by making glucose-1-phosphate the common reactant of two consecutive reactions which have a net negative free energy. Mechanisms of this type are apparently widely utilized in synthesis of polysaccharides, and perhaps in the synthesis of peptide linkages and in other condensation reactions involving the splitting out of water. Acyl phosphates may be the intermediates in many cases.

The mechanism of conversion of energy of phosphorylation to mechanical energy in muscle is currently the subject of intensive investigation (10, 11). Although details of the mechanism are unknown, it is clear that ATP reacts with actomyosin, and that energy of phosphorylation is utilized in converting the contracted form of this muscle protein complex into its extended form.

The mechanism of conversion of phosphorylation energy into osmotic energy is far from clear. It is not difficult to envisage mechanisms by means of which metabolites may be caused, by the expenditure of energy of phosphorylation, to move against a concentration gradient, but the actual mechanisms in use by organisms are thus far unknown. As an example of the general type of mechanism that may be employed, the following series of events may be postulated: On the left side of a membrane impermeable to glucose but permeable to glucose-6-phosphate, glucose reacts with ATP, yielding glucose-6-phosphate

mechanism, ATP/ADP ratios will become higher, and inorganic phosphate levels lower, than those corresponding to glycolytic equilibrium. The glycolysis process will then proceed toward equilibrium in a reverse direction, i.e., glycogen will be synthesized from lactic acid, with the utilization of three molecules of ATP and the production of three molecules of inorganic phosphate. This process will proceed until the lactic acid level falls to a value consistent with the low inorganic phosphate level and the high ATP/ADP ratio which obtain under aerobic conditions.

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Chapter XIII

THE CONTROL OF METABOLISM

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CONTROL OF METABOLISM

The literature on the physiological control of enzyme action is not voluminous, although many physiologists and enzymologists have long been aware that their respective efforts must be integrated if the living organism is ever to be understood.

The classical problem in the physiological control of enzyme action is the Pasteur effect, and on this subject the literature is abundant. Burk (1939) has presented a thorough discussion of the phenomenon, including an examination of the various definitions and the theories as to mechanism. In simplest terms, the Pasteur effect is the "oxygen inhibition of fermentative processes" and this in more specific terms means that in the presence of oxygen, less glucose will be converted to lactic acid than in the absence of oxygen. Johnson (1941) emphasized the role of inorganic phosphate in the mechanism of the Pasteur effect. He pointed out that inorganic phosphate is required for both oxidation and glycolysis and noted that oxidation could slow glycolysis if it could lower the intracellular phosphate to a point below the optimum level for the glycolytic process. Evidence that such a lowering could take place was also cited. Potter (1944) concurred in the conclusions drawn by Johnson and pointed out that the physiology of the whole organism must be integrated in terms of reaction components that are common to the several systems. He noted that the type of control advocated by Johnson might be basically applicable to physiological mechanisms of much broader interest than the Pasteur effect. Lipmann (1942) was inclined to doubt such simple explanations and to seek other "controlling factors". However, the task is so complex that while much is being done on the various isolated aspects of metabolism, knowledge of how these chemical reactions are integrated is still quite meager. No one has proposed that any single factor is the key to every problem of organismic control. But it is becoming more and more evident that the problem of organismic control is closely bound to if not identical with the problem of determining the course of the chemical reactions in the body, and the strategic importance of phosphate as a common factor in many biochemical systems is now thoroughly established. The present discussion will not attempt to de-emphasize the role of phosphorylation but will merely try to bring it into perspective with the many other factors that must also be considered in studies on the control of metabolism.

ALTERNATIVE METABOLIC PATHWAYS

Metabolism has for many years been considered in terms of catabolism (breakdown) and anabolism (synthesis), but it has not always been made clear that for large numbers of substances there are alternative paths of metabolism as Peters (1941) has pointed out. A given compound may be either broken down further to yield energy, or it may be combined with other compounds or chemical groupings to form a more complicated product needed for growth or function. What determines which pathway will be followed? Or, if several may be followed, what determines which pathway will predominate? If we consider an amino acid, for example, it is obvious that there are a number of alternative pathways that are known, and there are probably more to be discovered. Among the known pathways, we might include 1) conversion to peptides and protein 2) deamination to yield the corresponding α-keto acid, which can then be burned to CO_2 and water 3) decarboxylation to the amine 4) transformation to non-protein nitrogenous compounds. Less obvious, but equally significant, are the alternative pathways that confront some of the intermediates in carbohydrate breakdown. Pyruvate, for example, has many alternative pathways, as Barron (1943) has emphasized. Among the pathways we might include 1) reduction to lactate 2) oxidation to acetate 3) carboxylation to oxalacetate 4) condensation with oxalacetate to form a citrate precursor (see Green et al, 1948) 5) amination to form alanine 6) decarboxylation to acetaldehyde. The conversion of pyruvate to acetate is of interest because of the manifold reactions that this substance can undergo in the body. Bloch (1947) has reviewed the literature on this subject. Without entering the discussion as to whether the active metabolite is acetate or a derivative of acetate, it seems to be definitely proved that acetate can participate in the following reactions (1) acetylation of amines and amino acids (2) condensation to acetoacetate (3) condensation to fatty acids and (4) condensation to cholesterol, while other reactions have been suggested in addition.

While each of the reactions listed in the above 3 cases can be studied from the enzyme standpoint, one must ask what determines which reaction will occur in a given situation in vivo.

These few examples should suffice to illuminate the statement by Barron (loc. cit.) that, "the 'enzyme chemist' has not solved nor will he solve the chemical activities of the living cell if he does not constantly have in mind what the living cell is, and what controlling mechanisms are added to these cells as they go higher in their organization, in their evolutionary processes". The need for emphasizing these considerations is paramount in the study of cancer as Potter (1944, 1947) has pointed out, but they apply equally to all studies which seek an understanding of the function of living organisms, normal as well as abnormal.

How can the enzyme chemist bear in mind "what the living cell is" and "what controlling mechanisms are added"? It is certain that the answers to these questions are still largely unknown, and further that the answers to them will have to be provided by enzymologists. In attempting to follow Barron's admonition - as, indeed, we must - we can perhaps improve our position by setting up the categories of information that have to be integrated in order to provide an understanding of the cell. The problems can then be attacked separately, while the results can be seen in relation to the whole. The categories to follow have been developed in the course of the writer's teaching experience, and are not necessarily the only way that the problems could be classified.

Whether a given metabolite will follow this or that alternative metabolic pathway will depend in the first place upon whether the required enzyme is present. We must therefore consider the factors that control the <u>kind</u> of enzymes present in cells. Secondly, we must consider the <u>amount</u> of enzyme that is present. Third, we must consider the <u>location</u> of the enzyme, and finally, the most difficult of all, the consideration of the factors that control the enzyme <u>activity</u>. These 4 categories may now be briefly discussed.

CONTROL OF ENZYME OCCURRENCE*

That the enzyme pattern of a cell is the ultimate expression of its genetic constitution is unquestioned but it is also clear that other factors must be involved in the regulation of the enzyme content of the cell. Whether a given enzyme will depend on the presence of a given gene is not yet clear. The hypothesis that there is a one-to-one relationship between genes and enzymes has had a considerable heuristic value as is beautifully illustrated by the series of studies by Beadle and Tatum and their associates. One of the more complete accounts of the plan of these studies was presented by Beadle (1946), while additional material is available in other publications (Beadle, 1945a, b; Bonner, 1948; Horowitz et al, 1945; Tatum, 1944). This work was done with a mold (<u>Neurospora crassa</u>) which is able to grow on a "minimal" medium containing only inorganic salts, a source of energy and carbon atoms, such as glucose, a source of nitrogen such as nitrate or ammonia, and biotin. It can synthesize the remaining vitamins and all of the amino acids that are needed for synthesizing its proteins. Mold conidia (asexual spores) were treated with x-rays or ultraviolet light to produce mutations, and the spores were then crossed with receptive bodies of the opposite mating type from the original wild strain. From the fruiting bodies so obtained, individual spores could be isolated and grown on a "complete" medium containing amino acids and vitamins that the mutants might no longer be able to synthesize. Subcultures from the "complete" medium were then tested for growth in the "minimal" medium: if growth occurred no mutation involving any of the vitamins or amino acids had occurred, while if no growth occurred, it was apparent that a mutant had been isolated. In the latter case, the mutant could be tested on media lacking all of the amino acids or all of the vitamins, and if growth failed on either of these media the general type of mutation could be deduced. Further screening with media containing only one vitamin or amino acid could then be carried out and the exact nature of the mutation

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*The writer is indebted to Dr. J. Lederberg, Department of Genetics, University of Wisconsin, for suggestions in connection with this and the following section.

could be learned. Some 80,000 spores were tested in this way and some hundreds of mutants were isolated and maintained as stock cultures on the complete medium. Many of the mutants that were unable to synthesize given vitamins were found to be different from each other. In every case so far analysed, distinct genes have been involved in the control of distinguishable biochemical reactions, and where several genes have seemed to participate in a given transformation, it was plausible to postulate a distinct reaction corresponding to each gene. This work supports the postulate that there exists a one-to-one relation between chemical reaction and gene with the gene exerting its control over the chemical reaction by directly or indirectly controlling the production of specific enzymes. None of the work with Neurospora has yielded any evidence that would contradict the one-to-one concept (Bonner, loc. cit.) although it has been suggested that the experimental approach may tend to exclude other types of mutants, and further work may reveal cases in which the final specificity of an enzyme depends upon more than one gene. It is of course understood that many steps must be required to elaborate the various non-specific building blocks required for the final synthesis of an enzyme.

Evidence for the genetic control of enzymes based on direct determinations of enzyme activity is available in Neurospora, bacteria, yeasts, plants, and rabbits (Beadle, 1945a; Bonner, 1948; Monod, 1947). These experiments do not yet permit a final conclusion on the universal validity of the one-to-one concept.

There have been remarkable instances of biochemical pathways that are identical in Neurospora and in higher animals. For example, Neurospora can carry out the complete urea cycle that Krebs and Henseleit originally postulated for mammalian liver, involving the conversions through ornithine, citrulline and arginine. The mold can carry out many syntheses that are also carried out in the cells of mammals and there is reason to believe that the intermediate steps may be similar or identical in both cases. On the other hand, many compounds that can be synthesized by Neurospora cannot be synthesized by higher organisms, and are essential growth substances.

Thus the Neurospora work has been of tremendous significance not only in clarifying the relationship between genes and enzymes but also in helping to elaborate the alternative metabolic pathways that occur in living cells.

It is obvious that the most decisive way of controlling a given alternative pathway is by means of a mutation that will either delete the enzyme entirely, or introduce it in a cell in which it previously did not occur. But there are other means of control that are much more flexible, and in normal cells metabolic pathways are probably not altered by means of mutations, since for all practical purposes mutations are irreversible and furthermore relatively infrequent. On the other hand it seems quite possible that certain cancers are induced by mutations that alter the metabolic pathways of normal cells so as to produce cancer cells.

CONTROL OF ENZYME AMOUNT

A more subtle form of enzyme control is the regulation of the <u>amount</u> of each enzyme in the cell. Given two alternative metabolic pathways, with enzymes for both pathways present, the course of metabolism will depend in part upon the relative amounts of the respective enzymes. A discussion of this problem inevitably leads to a consideration of of the plasmagene concept, which may be stated at this point in the words of Spiegelman (1946) "enzymes may be produced by cytoplasmic components (plasmagenes) which possess the capacity for self-duplication". It may be noted that he also stated that "the evidence thus far presented does not permit a decisive conclusion as to whether the cytoplasmic self-duplicating unit is the enzyme itself or something mediating its formation". Spiegelman postulated that the plasmagenes are replicas of the nuclear genes, and that they are given off from the nucleus into the cytoplasm, where they control the <u>amounts</u> of protein and enzymes synthesized. An important aspect of the plasmagene concept is the idea of competition: "These plasmagenes, like all self-duplicating entities, compete with each other for protein and energy, and the outcome of such competitive inter-actions then determines the enzymatic constitution of the cytoplasm".

A question often raised is why there is a need for cytoplasmic entities with functions so similar to the functions of the nuclear genes. It has been suggested that the nuclear system provides for the persistence of the total enzymatic potentiality in heredity. However, such a system alone might be too inflexible to permit adaptive variation in the enzyme content of the cell. The latter need could be met by a system of self-duplicating cytoplasmic or nuclear entities capable of changing their absolute amount. If the entities are cytoplasmic they correspond to Spiegelman's plasmagenes; if nuclear, to gene lamellae such as proposed by Huskins (1947).

The fundamental observation that underlies these theories is the finding of differences in cells which by all known tests should have the same gene pattern.

The facts which Spiegelman sought to interpret involved the phenomenon of enzymatic adaptation in micro-organisms: "A population of cells placed in contact with some substrate acquires, after the lapse of some time, the enzymes necessary to metabolize the added substrate. The removal of the substrate leads to the disappearance of the enzyme system it evoked." It has been shown that the adaptation phenomenon occurred even in cells with a constant gene pattern ("genome") and in the absence of cell division, so that it could not be attributed to mutation and selection leading to a shifting population. These studies showed that the enzyme content of yeast cells could vary within wide limits in cells having the same gene pattern.

The second fact stressed by Spiegelman was that the kinetics of the increase in adaptive enzyme showed an increasing rate of enzyme formation, and a mathematical analysis of the data showed that it was compatible with the idea that the enzyme "is part of a cytoplasmic self-duplicating mechanism, such that, once the enzyme is formed, further formation of the enzyme molecules can proceed without the intervention of the gene". On the other hand, the observations are complicated by the fact that during the adaptation process, the conditions may have been such that as the amount of enzyme increased, the amount of energy available for the synthesis of more enzyme increased, so that the rate of enzyme production increased. The enzyme production might in this case be auto-catalytic kinetically without being due to self-duplicating cytoplasmic units (Monod, 1947, p. 246).

A third observation emphasized by Spiegelman was the fact that competition could be demonstrated between different enzymes undergoing simultaneous adaptive change (Spiegelman, 1946; Spiegelman and Dunn, 1947). When cells were exposed to both maltose and galactose, the maltose enzyme increased rapidly while the galactose enzyme increased slowly for 2 or 3 hours, following which the maltose enzyme decreased to about 1/3 of the maximum and the galactose enzyme increased more rapidly. It would appear that these facts are not readily explained simply in terms of total available energy.

Another observation of great significance was the finding that an enzyme could be maintained in cells that lacked the corresponding gene (Spiegelman, Lindegren and Lindegren, 1945). In this experiment the adaptive phenomenon was preserved in cells that lacked the gene for the given enzyme by maintaining the cells in the presence of the substrate throughout the process of mating, spore formation and subsequent development.

The studies mentioned above were carried out with micro-organisms. Other data are available in the case of mammalian tissues.

The various tissues in a higher organism are generally believed to retain the original gene pattern of the fertilized egg, and the current plasmagene theories are in general based upon this assumption (but cf. Huskins, 1947). The data may eventually lead to a better test of the assumption than is available at present. Some of the facts are as follows: Each organ in the animal body has a different pattern of enzyme distribution, both from the qualitative and quantitative standpoint, and each tissue within an organ has a specific enzymatic makeup (cf. Potter, 1944, 1947; Greenstein, 1947). Furthermore, a given tissue may undergo great changes in enzyme content in relatively short periods of time, with or without cell division. Studies on brain and liver from new born rats revealed marked changes during the first 15 days of life (Potter, et al., 1945). More decisive is the data on the concentration of various enzymes in the corpora lutea of rat

ovaries during pregnancy and lactation (Biddulph et al., 1946; Meyer et al., 1947; Stafford et al., 1947; McShan et al., 1948). The various enzymes increased and decreased at different rates during the period in which ovarian function was changing.

From these and other studies it is clear that the amount of enzyme in mammalian tissues may vary widely and it seems difficult to explain the variations as primary responses to specific genes. The ability of the organism to vary the amount of enzyme in a cell is obviously a much more delicate means of regulating alternative metabolic pathways than the simple addition or deletion of an enzyme. While the amount of enzyme might be varied by a mechanism in which individual genes formed homologous enzymes at varying rates the favored hypothesis is that genes form plasmagenes which in turn either form homologous enzymes or have enzymatic properties per se. Studies on enzyme distribution within the cell (Schneider, 1946) show that many enzymes are predominantly in the cytoplasm. This would seem to eliminate the hypothesis that nuclear-gene and enzyme are synonymous.

The variations in enzyme amount may perhaps best be explained by postulating that a cell contains two types of competitive autosynthetic bodies, the genes and the plasmagenes. The duplication of the former is somehow geared to cell division while the duplication of the latter is independent of cell division (cf. Potter, 1944). The amount of enzyme in a tissue would accordingly be affected by the relative rates of cell increase and of plasmagene increase. The amount of enzyme could fluctuate within wide limits but the changes would be reversible except in the extreme case in which cell division outpaced the division of some plasmagene to the point where the plasmagene became so "diluted" that some cells had none of it. Such a change could be irreversible if the homologous gene either did not exist or could not form the plasmagene, and would be difficult to distinguish from a "mutation". Model data for such phenomena have been obtained with Paramecia (Sonneborn, 1946; Preer, 1946).

CONTROL OF ENZYME LOCATION

The action of an enzyme will be influenced by the chemical environment (see next section) but this in turn will be determined in part by the location of the enzyme with respect to other enzymes. Methods and results in connection with the localization of enzyme activity within cells are given in the chapter by Schneider (p. 273), and are of unquestioned importance in the study of the living organism. While the genes must have much to do with the localization of enzymes (cf. Sinnott, 1946) unequal distribution of cytoplasmic components in embryonal development may be involved in subsequent tissue and structural differences. However little can now be said about the control of ultimate form and structure in terms of the relationship between genes and enzymes.

IN VITRO CONTROL OF ENZYME ACTIVITY

The knowledge of what enzymes are present in a cell, and the amounts that are present is only the beginning of an understanding as to what takes place in the cell. Many instances can be cited in which various enzymes in cells are present but not acting. The determination of the amount of enzyme in a cell would probably have no value whatsoever were it not for the fact that the amount of an enzyme is probably closely related to the extent of its use. Not only Spiegelman's data cited earlier but various other studies lead to the conclusion that when a given enzyme is not used in a cell, the absolute amount of the enzyme decreases.

Two generalizations may be made regarding the control of enzyme activity in cells. First, the activity is determined by the physico-chemical environment, and second, the environment is determined by the activity of all the surrounding enzymes and their location relative to the enzyme in question.

The factors of the physico-chemical environment may be considered as separate items, and usually are, in the in vitro tests made by the enzyme chemist. They are fairly well known and need not be considered in detail here.

Temperature increases the rate of enzyme activity within a narrow range that falls between 0° C. and about 60° C. Above a certain temperature that differs for various enzymes, the enzyme is irreversibly inactivated. Some enzymes are inactivated at temperatures as low as 40° while others will briefly withstand 100° C.

Hydrogen ion concentration has a profound effect on enzyme activity. Each enzyme has an optimum pH, which is one of its identifying characteristics. Some enzymes require an alkaline medium while others require an acid environment.

Substrate concentration is very important in regulating enzyme activity. For a considerable range of substrate concentrations, the activity of a given amount of enzyme is proportional to the amount of substrate. Increasing the substrate concentration beyond a certain point gives no further increase in activity.

Cofactor concentration may be decisive in determining enzyme activity, especially *in vitro*. The cofactors may be metal ions or metallo-organic compounds, or they may be derivatives of vitamins. In either case the enzyme is incomplete without the cofactor and if the cofactor is dissociable the activity is proportional to the amount of cofactor within a limited range.

Product concentration. Each enzymatic reaction has a fixed change of free energy ($\triangle F$) associated with it under standard conditions. If the energy change is small, the accumulation of the reaction product will slow the reaction, regardless of the amount of enzyme present; indeed the more enzyme the sooner the reaction will slow down. If the energy change is very large, the accumulation of the product will have virtually no effect on the rate of the reaction.

Inhibitor concentration. Each enzymatic reaction may be inhibited by numerous compounds that have structures similar to the substrate or that react with essential chemical groupings on the enzyme. In some cases the inhibition is reversible, while in other cases it is irreversible. The inhibition is proportional to the inhibitor concentration and may or may not be inversely proportional to the substrate concentration.

Oxidation-reduction potential. Most enzymes require a chemical environment that is neither strongly oxidizing nor strongly reducing. In either extreme, the enzyme is usually irreversibly inactivated (cf. Sizer and Tytell, 1941; Sizer, 1942).

Ionic strength. In addition to the specific activating effects of various inorganic ions, enzyme action may be profoundly affected by the ionic strength of the environment. In general, the activity is highest at the physiological level of ion concentration. Many anions and cations appear to be interchangeable, but no generalizations can be made. In certain instances the ratio of ions may be important in addition to the total concentration.

PHYSIOLOGICAL CONTROL OF ENZYME ACTIVITY

Each of the 8 aspects of the enzymatic environment listed above is in turn affected by the action of the other enzymes present. These are the factors that probably are of greatest importance in the actual regulation of metabolic pathways. The problem of the immediate future is to work out the mechanisms by which hormones on the one hand and changes in the external environment on the other, affect the chemical environment of specific enzymes.

Both Barron (1943) and Lipmann (1942) have emphasized the importance of controlling factors over and above the more obvious factors such as substrate concentration etc., and presumably they have had factors such as hormones in mind. We have taken the position (Potter, 1944) that the "inherent intracellular control which is the basis of life in primitive cells, is also the foundation upon which the specialized mechanisms must be laid. The 'controlling factors in the cell' must arise in response to metabolic events within the cell or in other cells". For example, one must discover how the basic metabolic system may be altered by inhibiting or activating some one of its enzymatic components with a hormone and one must inquire as to what metabolic product directly or in-

directly prompted the release of the hormone from the distant endocrine organ into the blood stream.

It has been suggested that the increase in oxygen uptake following an increased work output in a tissue, and the decrease in oxygen uptake soon after the onset of rest is readily explained in terms of the mechanisms of enzyme control that are already available (Potter, 1944). The stimulation was assumed to release a cofactor (possibly calcium) for ATP breakdown which furnished the energy for the work process and at the same time yielded products that stimulate glycolysis. Glycolysis would provide substrates for the oxidative enzymes, while both glycolysis and oxidation would reconvert adenylic acid and inorganic phosphate back to ATP. Fuel consumption would then decrease in rate due to a lack of phosphate and phosphate acceptor. Components of this system (e.g. lactic acid, CO_2, adenylic acid) were assumed to set off the bodily control mechanisms that are necessary for continued work performance or for integrating the needs of various organs. To emphasize such mechanisms does not deny the role of insulin, cortin, acetylcholine etc. but rather provides a background against which they must be examined. In the work cited, it was proposed that the growth mechanism was also geared into the same system.

SUMMARY

In conclusion it may be said that there is a very considerable area in which both the enzymologist and physiologist can afford to collaborate, and that there is a very great need for investigators who know something about each field, for certainly the two must be combined if we are to achieve a real understanding of living organisms. The enzyme chemist can determine what alternative metabolic pathways can occur in a given kind of tissue, but much more has to be done in order to know what reactions do occur. Studies on the living animal, using isotopically labelled metabolites, are perhaps the most attractive means now at our disposal for discovering what reactions actually do occur in the whole organism. However this approach is not self-sufficient. Many labelled compounds may never reach the site where they are acted upon when they arise in situ, due to permeability barriers, or more rapid metabolism at another site. We are inevitably drawn to the conclusion that every conceivable approach must be utilized and constantly evaluated against the other approaches if we are to unravel even a fraction of the mysteries of the living cell. While we cannot hope to reconstruct the cell, we can hope to understand the mechanisms that govern alternative metabolic pathways sufficiently to enable us to alter them by external means, i.e., by therapy in the case of the medical field, with more assurance than is now possible.

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Chapter XIV

THE DISTRIBUTION OF ENZYMES WITHIN THE CELL

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INTRODUCTION

Most enzyme studies in the past have dealt with the distribution of enzymes in different tissues. In general these studies have shown that although all tissues have qualitatively the same enzyme activity, on a quantitative basis the concentrations of the enzymes in the different tissues is widely different. Thus in quantitative terms, tissues can be said to have characteristic enzyme patterns. Obviously, since most tissues are composed of varying proportions of different types of cells, it is important to know which cells contain the enzyme activity and where the enzyme activity is localized inside the cell. Linderstrom-Lang (1938) and his associates have made outstanding contributions to the first problem in their classical correlations of enzymatic activities with the types of cells present in various tissues. The study of the intracellular localization of enzymes is the subject of the present chapter.

Before proceeding with the methods used for the study of the intracellular distribution of enzymes and the results that have been obtained, a brief description of the cytology of the cell is essential. It is now generally agreed that all cells consist of two major parts: A nucleus and a cytoplasm. The nucleus in turn contains the chromosomes, and one or more nucleoli, while the cytoplasm contains the Golgi apparatus, mitochondria, secretory granules, submicroscopic particles, and soluble material. Although other cellular inclusions have been recognized and named by the cytologist, they will not be considered in this chapter because no information about their enzymatic activity is available. (For further cytological information the reader is referred to the volume edited by Bourne, 1942.)

METHODS FOR THE DETERMINATION OF THE INTRACELLULAR DISTRIBUTION OF ENZYMES

Three methods have been used for studying the localization of enzymes within cells. Each method has its own advantages and disadvantages and these will be considered in the subsequent descriptions.

THE HISTOCHEMICAL METHOD

This method depends upon the formation of a product that can be seen directly under the microscope or that can be converted into a visible product. The histochemical method has been used for the study of the following enzymatic reactions: indophenol oxidase (cytochrome oxidase), peroxidase (formation of a colored product from benzidine in the presence of H_2O_2), dopa oxidase, acid and alkaline phosphatase, and lipase. These methods have recently been the subjects of reviews by Gomori (1946) and by Dempsey and Wislocki (1946). The main advantage of the histochemical method is the fact that the result of the enzymatic action can be seen in a single cell. This is important because it enables one to observe directly which cells of a tissue contain the enzyme in addition to revealing in what part of the cell the enzyme is localized. On the other hand, the site at which the product of the enzymatic action is seen may not be the site of the enzymatic reaction. Thus the product of the enzyme reaction may diffuse from one part of the cell and become adsorbed by another part of the cell that has a high affinity for the product. This criticism can be leveled at all the histochemical reactions, whether they are conducted on fixed or on fresh tissues. In addition, studies that involve the use of tissues that are subjected to fixation and embedding are subject to even more serious criticism inasmuch as these procedures may cause almost complete loss of enzymatic activity (Bodian, 1947; Stafford and Atkinson, 1948) or selective loss of enzymatic activity in certain parts of the cell (Bartelmez and Bensley, 1947). Obviously all the histochemical results and especially those obtained with fixed and embedded tissues must be accepted with considerable reserve.

MEASUREMENT OF ENZYMATIC ACTIVITY IN FRAGMENTS OF SINGLE CELLS

A more satisfactory method for the localization of enzymatic activity within cells involves the direct determination of enzymatic activity in single cells and in fragments obtained from single cells. This technic was developed and applied by Linderstrom-Lang and Holter and their co-workers (Linderstrom-Lang, 1938) and consisted essentially in scaling down current biochemical methods of analysis so that products produced by the enzymes in a fragment of a single cell could be measured. In their earlier work pieces of tissue were frozen and cut into thin sections. Alternate sections were then used for enzymic analysis and for quantitative histological analysis of the types of cells present. It was found that enzymatic activity could in certain cases be correlated with the number and type of cells present in a given tissue section. Thus the distribution of pepsin in the stomach mucosa was correlated quantitatively with the number of chief cells. Several other enzymes have also been localized within specialized cells of glandular tissues by this technic.

Subsequently it was found possible to extend these studies to single cells by applying these sub-micro biochemical methods of enzymic analysis to relatively large single celled organisms. Thus it was found that centrifugation caused sea urchin eggs to divide into two halves, one of which contained the nucleus and the other the mitochondria. Enzymic and cytological analysis of the two halves was then possible. In the case of the amoeba, centrifugation caused elongation of the cell and stratification of the cell contents. Holter and Doyle (1938) were able to divide the centrifuged amoeba into sections and correlate enzymatic activity of the sections with their mitochondrial content. In still another application of the Linderstrom-Lang technics, Duspiva (1942) determined the enzymic activity of measured quantities of nuclear material withdrawn with a micro-pipette from the nucleus of frog's eggs.

It is apparent that the technics of Linderstrom-Lang are rather limited in application. Thus the study of the intracellular distribution of enzymes has been limited to single celled organisms. The method has apparently not been applied to single cells of mammalian tissues. In addition it must be pointed out that the localization of enzymes within cells has depended on correlations of enzymatic activities with microscopically visible cell constituents. It would be of considerable interest to extend these studies to direct determinations of the enzymatic activities of cellular components isolated from single cells.

MEASUREMENT OF ENZYMATIC ACTIVITY IN FRACTIONS OBTAINED FROM LARGE NUMBERS OF DISRUPTED CELLS

The most versatile method for the study of the distribution of enzymes within cells is the so-called cell fractionation technic. In this method a piece of tissue is taken and the cells are broken mechanically (mortar or homogenizer) thus releasing the cell contents. The different parts of the cells can then be isolated by the application of gradually increasing centrifugal force. Since the nuclei are the largest in size of the cell components and the most dense, they are sedimented at low speeds. At higher centrifugal forces the mitochondria sediment, followed at still higher speeds by the submicroscopic particles. If the nuclear membranes are broken by the use of fine sand or the Waring blendor, the chromosomes are released and can also be isolated by differential centrifugation. For details of the isolation procedures, the reader is referred to the papers by Dounce (1943), Claude (1941, 1943, 1944, 1946), Mirsky and Ris (1947), and Hogeboom, Schneider and Pallade (1948). One advantage of the cell fractionation technic is that almost any amount of a particular fraction can be prepared in pure form. Thus the number of enzyme reactions that can be studied is limited only to the diversity of enzymatic reactions known to current biochemistry, provided of course that adequate assay methods are available or can be developed. Although a large number of enzymes can be studied with this technic, the method has several disadvantages. In the first place since pieces of tissue are used, more than one type of cell is being studied. This limitation can be avoided by the choice of tissues which are composed almost exclusively of one type of cell. Furthermore the mere act of breaking the cell membranes must be considered as a

source of artifacts such as adsorption or release of enzymes or other materials by the particulate components of the cell, or the production of particulate components by cell rupture. These phenomena of adsorption and release of enzymes can be tested by washing the isolated particulate components of the cell in different solutions and the identity of the isolated material can be established by careful cytological observations.

From these brief discussions of the methods for the study of the intracellular distribution of enzymes, it is apparent that no one method is free of limitations or disadvantages. It is also apparent that objections that can be lodged against the use of one method can be overcome by the application of one of the other methods. It is to be hoped that research will be designed that will provide information on the same enzymes with several methods.

ENZYMATIC ACTIVITY ASSOCIATED WITH THE NUCLEUS

Using the histochemical technic for the demonstration of phosphatase activity, some interesting observations have been made. Willmer (1942) observed marked alkaline phosphatase activity in the chromosomes of cells in tissue culture. Deane and Dempsey (1946) observed that the localization of phosphatase activity in mouse duodenum was highly dependent on the substrate used as well as on the pH of the reaction mixture. They found that the nucleolus exhibited prominent lecithin phosphatase activity at an acid reaction while the chromosomes of dividing cells showed pronounced glucose-1-phosphatase activity at an alkaline reaction. Krugelis (1946) studied alkaline phosphatase activity with glycerophosphate and partially depolymerized nucleic acids as substrates and observed that with desoxyribonucleic acid the salivary gland chromosomes of Drosophila showed activity in the same regions as desoxyribose nucleic acid was localized.

Duspiva (1942) used a unique technic for the study of the dipeptidase activity of nuclei. He withdrew measured amounts of material from the nucleus with a micropipette and measured the activity of the aliquot towards alanylglycine with the submicro technics of Linderstrom-Lang and Holter. He found that the activity of the enzyme in the nucleus was low as compared to the cytoplasm of the eggs of Rana temporaria while in the case of the eggs of Rana esculenta the activity of the nucleus was about the same as that of the cytoplasm.

Behrens (1939) isolated nuclei from powders of liver that had been frozen and dried in the frozen state. This was done by suspending the powder in a mixture of organic solvents and centrifuging. It was found that the arginase concentration of the nuclei was about the same as that of the cytoplasm while the lipase concentration in the nuclei was very low as compared to that of the cytoplasm. Dounce (1943) used an entirely different technic for the isolation of liver nuclei. He froze the liver and then broke the liver cells in the Waring Blendor in a solution of dilute citric acid. The liberated nuclei were then isolated by differential centrifugation. It was found that the arginase concentration in the nuclei was about 45 per cent as great as in the whole liver. Cytochrome oxidase, esterase, the apoenzyme of lactic dehydrogenase, and acid phosphatase also were present in similar concentrations in the isolated nuclei while the concentration of alkaline phosphatase was about 2 times as great as in the whole liver. In addition Dounce found that catalase, succinic dehydrogenase, cytochrome c, coenzyme I, and riboflavin were either absent or present in very small amounts in the liver nuclei. Lan (1943, 1944) extended these studies to d-amino acid oxidase and uricase. These enzymes were found to be present in the nuclei in the same or slightly higher concentrations than in whole liver. More recently Dounce and Beyer (1948) have observed that nuclei isolated from liver or kidney had an aldolase activity which was about 40 per cent as great as that of the whole tissues. Euler et al (1945) using the method of Dounce for the isolation of nuclei were able to demonstrate succinic and lactic dehydrogenase activity in isolated rat liver nuclei with the Thunberg technique. Schneider (1946) fractionated rat liver into a nuclear fraction, a large granule or mitochondria fraction and an unfractionated residue. The nuclear fraction contained all of the nuclei of the original liver tissue, but also contained some large granules. It was found in agreement with the results of Dounce (1943) that the concentration of cytochrome oxidase was 53 per cent as great in the nuclear fraction as in the whole tissue. It was also pointed out however, that this concentration of enzyme accounted for only about 5 per cent of the total cytochrome oxidase

activity of the liver tissue and that the major portion of the cytochrome oxidase activity of the liver tissue was recovered in the large granule fraction in which the cytochrome oxidase concentration was 416 per cent as great as in the whole liver. It may be concluded that the significance of the association of these enzymes with the nuclei is obscure inasmuch as the concentrations of the enzymes in the nuclei was (with the exception of alkaline phosphatase) lower than in the whole tissue.

ENZYMATIC ACTIVITIES ASSOCIATED WITH THE CYTOPLASM

THE GOLGI APPARATUS

Emmel (1945) discovered that the supranuclear region of epithelial cells of the small intestine showed marked alkaline phosphatase activity. This region corresponded closely to that of the Golgi apparatus. This observation was confirmed and extended by Deane and Dempsey (1946) who found that the Golgi region of other cells exhibited marked phosphatase activity, both at alkaline and acid reaction. Further advances in the study of the Golgi material will undoubtedly await the isolation of this material by differential centrifugation.

THE MITOCHONDRIA

No attempt has apparently been made to determine whether enzymatic reactions could be demonstrated in the mitochondria by histochemical technics. The histochemical methods have however demonstrated enzymatic reactions associated with granules located in the cytoplasm. Whether these granules are related to or identical with the mitochondria will await further research. The association of oxidases, peroxidases, and lipases with these cytoplasmic granules has been reviewed recently (Joyet-Lavergne, 1938 and Dempsey and Wislocki, 1946) and need not be discussed further here.

Holter and Doyle (1938) studied the distribution of amylase in centrifuged specimens of Amoeba proteus. These authors cut the centrifuged amoebae into several sections and determined the amylase activity and the number of mitochondria in each section. They found that the localisation of amylase followed most closely the distribution of mitochondria and concluded that the enzyme was probably bound to the mitochondria in this organism.

The largest number of studies on the association of enzyme activity with mitochondria has been made on the mitochondria isolated from large numbers of cells by centrifugation. The first such study was made by Warburg in 1913, who found that the oxygen uptake of granules obtained by centrifugation of cell free guinea pig liver extracts accounted almost entirely for the oxygen uptake of the extract. Lazarow and Barron (1943) made similar studies, but found that mitochondria, nuclear concentrate, and supernatant all absorbed oxygen. The data of these workers is expressed only however as Q_{O_2} values and it is impossible to determine what fraction of the oxygen uptake is associated with each fraction. These workers also observed that there was a considerable augmentation of the oxygen uptake when succinic acid was added to the nuclear fraction or to the mitochondria fraction, when glutamic acid was added to the mitochondria, and when ethyl alcohol was added to the supernatant. Brachet and Jeener (1944) centrifuged cell free extracts of a large number of tissues at high speed and found that a large number of enzymes was partly bound to the sedimented particles which undoubtedly included both the mitochondria and the submicrospic particles. The enzymes included acid and alkaline phosphatase, amylase, dipeptidase, cathepsin, trypsin, arginase, and adenylic acid deaminase. Chantrenne (1944) in a similar study with yeast extracts found that the granules sedimented from the yeast extract contained cytochrome oxidase, cytochromes a and b, succinic dehydrogenase, and peroxidase. Herrmann and Boss (1945) isolated the pigment granules of the ciliary body and found that these granules possessed cytochrome oxidase, succinoxidase, and dopa oxidase activity. It is rather interesting that these pigment granules should possess enzymatic activity since, as these authors point out, they have generally been assumed to be metabolically inert. In 1945, Steinbach and Moog isolated cytoplasmic particles from chick embryos and observed that they had high adenylpyrophosphatase activity. The isolated particles were apparently a mixture of both mitochondria and submicroscopic par-

ticles as was the case in the work of Brachet and Jeener and Chantrenne. In the work described so far, no apparent effort was made to determine whether the total enzyme activity in the fractions equaled the total activity in the whole tissue. As a consequence the results are open to serious criticism because it may be that in the original tissue activators or inhibitors are present which may or may not have the same distribution in the cell as the enzyme system under consideration. This criticism can be met by demonstrating that the sum of the activities in the isolated fractions equals the activity of the original tissue. This was first done by Schneider (1946) who separated rat liver, rat kidney, and rat liver tumor into a nuclear fraction, a large granule or mitochondria fraction, and an unfractionated residue, and studied the distribution of succinoxidase, cytochrome oxidase, and adenosinetriphosphatase in these fractions. The validity of the assay methods was indicated by the fact that the total enzyme activities recovered in the fractions agreed closely with the total activities of the original tissue suspensions. The enzymes succinoxidase, cytochrome oxidase, and adenosine triphosphatase were found mainly in association with the mitochondria fraction of rat liver and kidney. This was also true of the tumor with the exception that the adenosinetriphosphatase was associated mainly with the unfractionated residue. At the same time, Hogeboom, Claude and Hotchkiss (1946) reported similar results with rat liver. They found that essentially all of the succinoxidase and cytochrome oxidase activity of cell free rat liver extracts was associated with the large granules. Subsequently Hogeboom, Schneider and Pallade (1947) improved the technic for the isolation of mitochondria and submicroscopic particles and presented strong cytological evidence in support of the view that the isolated particles were actually mitochondria. As a result it was necessary to re-examine the previous results. These studies (Hogeboom, Schneider and Pallade, 1947, 1948, and Schneider, 1947) showed that a major portion of the succinoxidase activity of the liver cell was associated with the isolated mitochondria. Graffi and Junkman (1946) isolated both mitochondria and submicroscopic particles in a single fraction from rat liver and carcinoma cells and observed that this fraction was able to reduce methylene blue in the presence of either succinate or lactate. The nuclear fractions and the soluble protein fractions obtained from these tissues had only faint activity toward these substrates. Chantrenne (1947) extended his previous results to a study of the distribution of alkaline phosphatase and adenosinetriphosphatase in mouse liver. He prepared a cell free extract of the tissue and then isolated 5 fractions of particles of decreasing size. He found that all the fractions possessed enzyme activity, but that the larger particles had higher enzyme concentrations than did the smaller particles. Again no data was presented to show that the total enzyme activity of the fractions equalled that of the original tissue. The distribution of cytochrome c in rat liver fractions was studied by Schneider, Claude, and Hogeboom (1948). These authors observed that a large percentage of the cytochrome c present in a suspension of liver which had been cleared of cells and nuclei was associated with the mitochondria. Moreover, the cytochrome c was retained by the mitochondria after several washings and was biologically active as shown by the fact that the oxidation of succinic acid occurred at 60 per cent of the maximum rate obtained in the presence of excess added cytochrome c. The cytochrome c which was not associated with the mitochondria was apparently in a soluble form since it was not associated with the submicroscopic particles. A preliminary report by Kennedy and Lehninger (1948) indicates that the enzyme complex that oxidizes fatty acid is also associated with the mitochondria. Schneider (1948) has independently obtained similar results. Price, Miller and Miller (1948) studied the distribution of riboflavin in fractions obtained from the livers of rats on low and high riboflavin diets. They found that about 50 per cent of the liver riboflavin was associated with the mitochondria and this was the only fraction in which the riboflavin concentration was higher than in the whole liver. The increased riboflavin found in the livers of the rats on the high riboflavin diet was paralleled by a corresponding increase in the riboflavin content of the mitochondria of these livers. Claude (1944b) has also stated that α-glycerophosphate dehydrogenase, d-amino acid oxidase and ribonuclease are associated exclusively with the mitochondria of rat liver. These results have never been published in detail.

THE SECRETORY GRANULES

Van Weel and Engel (1938) demonstrated that there was a striking correlation between the granule content of pancreatic cells of the mouse and the carboxypolypeptidase activity. These authors used the serial sectioning methods of Linderstrom-Lang (1938) and were able

to show that the injection of pilocarpine into fasting mice resulted in a marked drop in the granule content of the pancreatic cells followed by a gradual increase in the granule content until at the end of about 12 hours the granule content had returned to the original level. These changes in the granule content of the pancreas were closely paralleled by the carboxypolypeptidase activity of the pancreas thus indicating that this enzyme was associated with the granules that were being secreted by this gland in response to the pilocarpine injection.

The large granules isolated from liver cells by differential centrifugation were at first considered to be secretory granules (Claude, 1943). Subsequently this view was modified and it was stated that the large granules were a mixture of mitochondria and secretory granules (Claude, 1946). More recently (Hogeboom, Schneider, and Pallade, 1948) found that apparently all the particles isolated in the large granule fraction were mitochondria as demonstrated by vital staining with Janus Green B and presented evidence that the secretory granules were apparently disintegrated when the cells were broken. In the case of other tissues however it is not certain that all the elements isolated are mitochondria. This would appear to be especially true of the pancreas since the secretory elements of this tissue seem to be more stable. In this connection it is of interest to note that Brachet and Jeener (1944) observed that one of the secretion products of the pancreas, trypsin, was associated with the particulate material isolated from this tissue.

THE SUBMICROSCOPIC PARTICLES

Omachi, Barnum, and Glick (1948) made a careful study of the distribution of esterase in mouse liver homogenates. These authors found that 47 per cent of the esterase activity of the original liver was associated with the submicroscopic particles and that the esterase concentration in this fraction was 4.2 times as great as in the homogenate. Hogeboom (1949) has studied the distribution of cytochrome c reductase in rat liver fractions. He found that 48 per cent of the reductase activity of the homogenate was associated with the submicroscopic particles and that the specific activity of the submicroscopic particles was about 3 times as great as that of the homogenate. The enzymes esterase and cytochrome c reductase are the only ones that have been demonstrated to reach high concentrations in the submicroscopic particle fraction.

ENZYMATIC ACTIVITY NOT ASSOCIATED WITH PARTICULATE MATERIAL

The enzymes in this category include all the soluble enzymes. However, the fact that an enzyme has been obtained in soluble form does not necessarily indicate that the enzyme is present in a soluble form in the cell. In the previous sections several soluble enzymes have been demonstrated to be associated with insoluble particulate material. As a result it will be necessary to test each enzyme separately to determine whether or not it is actually present in the cell in soluble form. Several enzymes appear to be present in the cell in soluble form. Thus Claude (1944b) has stated that transaminase and malic dehydrogenase were probably present in the liver cell in soluble form. Chantrenne (1944) found that catalase, lactic dehydrogenase, carboxylase and cytochrome c were present in the supernatant remaining after the removal of particulate material from yeast extracts. Schneider, Claude and Hogeboom (1948) also observed that a considerable portion of the cytochrome c of rat liver was not associated with either the mitochondria or the submicroscopic particles.

ENZYMATIC ACTIVITIES WHOSE LOCALIZATION WITHIN THE CELL IS UNCERTAIN

Linderstrom-Lang (1933), Philipson (1935), Holter (1936), Holter and Kopac (1937), and Doyle (1938) demonstrated that the peptidase and catalase activities of certain marine eggs were not associated with the mitochondria of these cells but that these enzymes appeared to be localized within the hyaline ground substance of the cytoplasm of these cells. It is not clear however, whether these enzymes are bound to submicroscopic particulate material in the cytoplasm of these cells or whether they are present in soluble form in the cytoplasm. Engel and Bretschneider (1947) have studied the distribution of

amylase, proteinase, and esterase and of mitochondria in various tissues of the wheat grain. They found that there was no correlation between the number of mitochondria in a tissue and the enzymatic activities.

Zittle and Zitin (1942) disintegrated bull spermatozoa sonically and separated the broken cells into heads, midpieces and tails by means of differential centrifugation. They found that the highest concentration of cytochrome oxidase was found in the tails and the lowest concentration in the heads which contained the nuclei. It must be pointed out however, that the concentration in the tails was only slightly higher than in the uncentrifuged broken cell suspension. Since the recovery of enzymatic activity in the fractions was not determined the significance of the results is somewhat questionable.

Several recent enzymatic studies have involved the use of insoluble residues obtained by low speed centrifugation of liver or kidney homogenates. These residues appear to be mixtures of all of the nuclei of the homogenate and 40-80 per cent of the mitochondria (Schneider, 1946; Hogeboom, Schneider, and Pallade, 1948; Price, Miller and Miller, 1948). The enzymatic reactions that these residues catalyze include the oxidation of fatty acids (Lehninger, 1945), the oxidation of the acids of the Krebs tricarboxylic acid cycle (Green, Loomis and Auerbach, 1948), oxidative phosphorylation (Loomis and Lipman, 1948) the synthesis of p-amino hippuric acid (Cohen and McGilvery, 1947), and the synthesis of citrulline from ornithine (Cohen and Hayano, 1948). Recently Kennedy and Lehninger (1948) and Schneider (1948) independently demonstrated that the first of these reactions, the oxidation of fatty acids, was associated exclusively with the mitochondria. No attempt has been made to localize the site of the other enzymatic reactions more precisely.

CONCLUSION

It is apparent from this discussion that much remains to be learned about the distribution of enzymes within cells. The method that appears to have the widest applicability at the present time is the cell fractionation technic. It is to be hoped that in future applications of this technic more attention will be paid to the necessity for assaying all fractions obtained for enzymatic activity than has been done in the past. In addition it is to be hoped that a greater effort is made to control the fractionation of broken cell suspensions with careful cytological observation as well as to devise independent methods whereby the results obtained by the cell fractionation technic can be tested. The tools for a concerted attack on the intracellular distribution of enzymes are at hand. Much depends upon their proper application.

Although the data available at present is far from complete, the over-all picture that can be obtained is that the mitochondria appear to be the most important center of metabolic activity in the cell. As more and more enzymes are studied, the function of the other particulate components of the cell should become clear.

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